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INVESTIGATING THE SIGNALLING AND FUNCTIONAL ACTIVITY OF CD24 IN CANCER

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Declaration

I declare that this work is a result of my own work throughout the period of study at the University of Nottingham except where otherwise stated in the text, and that no part of this work has been submitted, whether in the same or a different form, to this or any other university for any degree other than for which I am now a candidate.

HASSAN OTIFI

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Dedication

I dedicate my thesis work to my mother, wife, and children. My greatest gratitude to my wife, I cannot thank her enough for the incredible support, love, patience, and sacrifices she made throughout my Ph.D. program. I also dedicate this work and give special thanks to my wonderful sons and daughters for being there for me throughout the entire period of my Ph.D.

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Abbreviations

2-ME	2-mercaptoethanol
5-FU	5- fluorouracil
ABD	Actin-binding domain
ACF	Aberrant crypt focus
ADC	Adenocarcinoma
APC	Adenomatous polyposis coli
ARHGEF	Rho guanine nucleotide exchange factor
BCA	Bicinchoninic acid
bp	Base pairs
BSA	Bovine serum albumin
CD24	Cluster of differentiation 24
CFE	Colonies formation efficiency
CGH	Comparative genomic hybridization
CHX	Cycloheximide
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
Co-IP	Co-Immunoprecipitation
CRC	Colorectal cancer
CSCs	Cancer stem cells
Cten	C-terminal tensin-like
CXCR	Chemokine receptor type
DAB	3, 3‟-Diaminobenzidine tetrahydrochloride
DAMPs	Damage-associated molecular patterns
DAPI	4', 6-Diamidino-2-phenylindole
DCC	Deleted in colorectal cancer
DCs	dendritic cells
DLC	Deleted in liver cancer
DLG	Drosophila discs large

DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DN-TCF	Dominant-negative T-cell factor
dNTP	Deoxyribonucleotide triphosphate
Dok	Downstream of tyrosine kinase
DTT	Dithiothreitol
E	Exon
E.coli	Escherichia coli
E-cad	E-cadherin
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinase
FAB	Focal adhesion-binding
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FAMMM	Familial atypical multiple mole melanoma
FAP	Familial adenomatous polyposis
FAs	Focal adhesions
FBS	Foetal calf serum
Fc	Fagment crystallizable
FGFR	Fibroblastic growth factor receptor
FISH	Fluorescence in situ hybridization
GFP	Green fluorescent protein
GIT	Gastrointestinal tract

GPI	Glycosyl phosphatidylinositol
HAS	Heat-stable antigen
HCC	Hepatocellular carcinoma
HDACs	Histone deacetylases
HER	Human epidermal growth factor receptor
HEST	Human embryonic stem cells
HNPCC	Hereditary non-polyposis colorectal cancer
HP	Hereditary pancreatitis
HPRT	Hypoxanthine phosphoribosyl transferase
HRM	High Resolution Melt
HRP	Horseradish peroxidase
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IHC	Immunohistochemistry
IL	Interleukin
ILK	Integrin-linked kinase
IVT	In vitro transcription
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Kb	Kilo bases
kDa	Kilo Dalton
KIFAP	Kinesin superfamily–associated protein
KRAS	Kirsten rat sarcoma viral oncogene homolog
LB	Luria broth
LEF	Lymphoid enhancer factor
LN_2	Liquid nitrogen
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
Luc	Luciferase
MAPK	Mitogen-activated protein kinase

MDCK	Madin Darby Canine Kidney
MDM	Murine double minute
MFI	Mean fluorescence intensity
MLCSV40	Immortalised human prostatic epithelial cell line
MLH	MutL homolog
M-MLV	Murine leukaemia viruses
MMP	Matrix metalloproteinases
MMR	Mismatch repair
mRNA	Messenger RNA
MSH	MutS homolog
MSI	Microsatellite instability
MSS	Microsatellite stable
MTORC	Mammalian target of rapamycin complex
N-cad	N-cadherin
NLS	Nuclear localisation signal
NSCLC	Non-small cell lung cancer
NTC	No template control
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Pancreatic cancer
PCR	Polymerase chain reaction
p-CREB	Phospho-cAMP responsive element binding protein
PDCD	Programmed cell death
PDK	Phosphatidylinositol-Dependent Kinase
PDM	Pre-diagnostic multiplex
PE38	Pseudomonas exotoxin derivative
p-eNOS	Phospho-Endothelial NOS
p-FAK	Phospho-FAK
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol kinase 3 catalytic

PINCH	Particularly interesting Cys-His-rich protein
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PJS	Peutz-Jeghers syndrome
p-MAPK	Phosphorylated mitogen-activated protein kinase
p-mTOR	Phospho-mammalian target of rapamycin
PR	Progesterone receptor
PRL	Protein tyrosine phosphatase
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
QMC	Quick-Multiplex-Consensus
qPCR	Quantitative polymerase chain reaction
Rac	Ras-related C3 botulinum toxin
RalA	Ras-related protein Ral-A
RalB	Ras-related protein Ral-B
Rec.	Recombinant
RhoA	Ras homolog gene family, member A
RIN	RNA integrity number
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
T-ICs	Tumour-initiating-cells

Abstract

Background and Aim: The cluster of differentiation 24 (CD24) is a human protein encoded by the CD24 gene which maps to chromosome 6q21. It is a small highly-glycosylated protein that is attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. Various studies have proven that CD24 is overexpressed in many human tumours, and its expression level has been found to be associated with a poor prognosis. Recently, CD24 has also been identified as a stem cell marker in several types of cancer. However little is known about CD24's biological role in cancer or the mechanism through which it acts in cancer development. Thus, the main aim of this study is to investigate the potential functional role and signalling pathways of CD24 in various cancer models, such as colorectal cancer (CRC), pancreatic cancer, liver cancer, and lung cancer.

Methods: The signalling pathway and functional role of CD24 has been studied in a total of 13 well-characterised cell lines from four cancer models i.e. cancers of colorectum, pancreas, liver and lung. To identify potential downstream targets of CD24, CD24 was forcibly expressed via transient transfection in cell lines expressing low levels of CD24 or, in contrast, it was knocked down using RNA interference (siRNA) in cell lines expressing high levels of CD24. Subsequent to CD24 manipulation, we used Western blotting and/or qPCR to investigate changes in the expression of specific proteins that have been hypothesised to be involved downstream in the CD24 signalling pathway e.g., C-terminal Tensin-like (Cten), focal adhesion kinase (FAK), integrin-linked kinase (ILK) and Src. In addition, changes in epithelial mesenchymal transition (EMT) markers were evaluated. Similarly, in order to find potential upstream regulators of CD24, the expression levels of some proteins that have been found to be associated with cancer (e.g., KRAS and EGFR) were manipulated using specific siRNAs or inhibitors/stimulators, and the changes in CD24 expression were then evaluated. The functional effect of these interventions were tested through measuring cell motility,

invasion, proliferation, and stemness (by the colony-formation in agar). Physical interaction between proteins was tested by protein complex immunoprecipitation (CO-IP) and protein stability was tested using cycloheximide (CHX). Lastly, the subcellular localisation of CD24 was studied in CRC and lung cancer cell lines and, immunohistochemically, in tumour tissues of CRC (n=84) and NSCLC (n=58).

Results: Our data have shown that manipulating of the expression of CD24 in the tested cancer model cell lines resulted in a significant change in the expression level of Cten which in turn caused changes in the expression levels of ILK and FAK. Noticeable modifications to cell migration, invasion, proliferation, and colony-forming rate (all $p<0.05$) following CD24 manipulation have also been detected, indicating that the up-regulation of Cten, ILK and FAK expression by CD24 may reveal the mechanism via which cell functions are regulated. The up-regulation of Cten expression by CD24 was found to be due to protein stabilisation as confirmed by qPCR and CHX assy. CD24 was observed to activate AKT at Serine 473 (Ser473), rather than at the Threonine 308 (Thr308) residue, and potentially collaborate with PI3 kinase to induce the full activation of AKT. The inhibition of EGFR using a specific EGFR inhibitor, PD135053, and the stimulation of EGF using recombinant EGF in cell lines that did not harbour mutant KRAS resulted in significant modifications to CD24 expression, as well as in cell motility, suggesting that EGFR is an upstream regulator of CD24 expression. However, an inverse association between CD24 and KRAS was observed suggesting that EGFR does not signal to CD24 through KRAS. The signalling pathway would appear to be EGFR → CD24 →Cten →ILK/FAK→ AKT. These effects were seen in all of the models tested thereby confirming the role of CD24 in many cancers. In CRC and NSCLC cell lines and tissues, CD24 was found to be localised in both the cytoplasm and in the nucleus. An association between CD24 and its associated partners, including downstream targets, was observed in tumour tissues. This association was consistent with that observed following CD24 manipulation in cancer cell lines.

Conclusion: CD24 seems to be regulated by EGFR either directly or indirectly. Consequently, it regulates Cten, FAK and ILK and enhances cell motility, invasion, proliferation and stemness in various cancer model cell lines. A combination of Anti-CD24 antibody/siRNA and PI3 kinase inhibitors could be used in clinical practice as a potential therapeutic agent in the early stages of CRC. Our observations in the four cancer models were consistent, suggesting that CD24 may regulate cell functions in these models through a unique mechanism. The association between nuclear CD24 expression and cancer progression and metastasis should be explored in further studies.

Thesis-Related Publications and Conference Communications

A. In process publications

OTIFI, H. and ILYAS, M., CD24 regulates cell function activities through stabilisation of Cten expression level (manuscript in preparation)

OTIFI, H., AL-GHAMDI, S, and ILYAS, M., CD24 is up-regulated by EGFR pathway to modulate cell function in colorectal and lung cancer (manuscript in preparation).

OTIFI, H. and ILYAS, M., CD24 regulates cell functions in various cancer models via a unique mechanism (manuscript in preparation).

B. Presentations and Conference Communications

OTIFI, H. and ILYAS, M., 2014, Investigating the Biological Role of CD24 in the Development of Colorectal Cancer. Virchows Archiv, 465, S323-S323. 26th European Congress of Pathology, 30 August - 3 September 2014, London, UK

OTIFI, H. and ILYAS, M., 2014, CD24 Regulates Cten, FAK and ILK, and Enhances the Cell Migration and Proliferation in Colorectal Cancer. NCRI Cancer Conference, Liverpool 2 – 5 November 2014

OTIFI, H. and ILYAS, M., 2015, The Significance of CD24 Gene in the Development of Colorectal Cancer. The 8th Saudi Students Conference, Imperial College, London 31 January –1 February 2015

OTIFI, H., AHMED, M., AL-GHAMDI, S., and Ilyas, M., 2016, CD24 Modulates the Focal Adhesion Molecules Pathways and Cell Functions by Signalling Through TNS-4 in Colorectal Cancer. The 8th Saudi Students Conference, University of Birmingham, 13 -14 February 2016

OTIFI, H. and ILYAS, M. I., 2016. CD24 is Significantly Involved in Pancreatic Cancer Progression by Targeting Focal Adhesion Molecules and Regulating Cell Functions JOURNAL OF PATHOLOGY. 240, 33-33- Nottingham Pathology 2016 Meeting, 28 June – Friday 01 July 2016, Nottingham, UK.

OTIFI, H. and ILYAS, M. I., 2016. CD24 Modulates Focal Adhesion Molecules and Regulates Cell Functions in Non- Small Cell Lung Cancer JOURNAL OF PATHOLOGY. 240, 32-32- Nottingham Pathology 2016 Meeting, 28 June – Friday 01 July 2016, Nottingham, UK.

THORPE, H., ASIRI, A., **OTIFI, H**. and ILYAS, M., 2016. Cten Knockout Using CRISPR/Cas9 Abrogates Cell Migration and Invasion in Colorectal Cancer Cell Lines JOURNAL OF PATHOLOGY. 240, 13-13- Nottingham Pathology 2016 Meeting, 28 June – Friday 01 July 2016, Nottingham, UK.

SAIRA, S. S., **OTIFI, H. O. HASSAN**, GHAMDI, S. G. SALEH, AKHLAQ, M. A. MAHAM and ILYAS, M. I., 2016. CTEN is Downstream Target of CD24 JOURNAL OF PATHOLOGY. 240, 33-33- Nottingham Pathology 2016 Meeting, 28 June – Friday 01 July 2016, Nottingham, UK.

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1 GENERAL INTRODUCTION

1.1 Cancer

Cancer is an evolutionary disease, following the model of an asexual reproducing unicellular organism, leading to progressive acquisition of features /hallmarks of cancer (Makohon-Moore and Iacobuzio-Donahue, 2016). Changes in behavioural features of cancer cells, including loss of controlled cell growth, division, and apoptosis, among the main features of cancer development (Parsons et al., 2008). In several cases, proliferation of cancer cells lead to enlargement of a tumour, and the cells which can spread to other parts of the body where they can develop secondary tumours - a process known as metastasis. There are two main categories of tumours - benign tumours (local, do not spread) and malignant tumours (spread and usually invasive). Despite the fact that benign tumours do not spread to further organs, they can grow enormously but are typically not life-threatening (Fisher, 2009). In contrast, cancer is considered a main cause of mortality worldwide, and although a huge amount of effort and economics resources are put towards treatment, the suppression or control of this progressive disease remains a great challenge for clinicians and researchers (Jemal et al., 2008).

The phenotype of cancer includes a wide range of properties that together generate the cancer clinical entity. When matching cells and tissues that are derived from cancer patients with those of healthy subjects, or in even certain cases, with histologically normal tissue from the same person, it is necessary to catalogue their various changes in molecular, cellular, and biological characteristics. The latest application of transcriptional profiling to cancer has identified differences in the expression of a huge number of genes as normal cells undergo transformation into their malignant derivatives. A number of these

modifications in expression are shared by many types of cancer cells, while others appear to be reserved to only one or a small subgroup of tumour cell types encountered in the oncology clinic (Hahn and Weinberg, 2002). A thorough knowledge of the intricate interactions between biological, clinical, genomic, and societal factors, in addition to lifestyle, is essential to better understand cancer development. Researchers have found that cohort studies help to better comprehend cancer aetiology, study cancer results, improve risk prediction analyses and models, and develop rules for control and prevention strategies (Tangen, 2016).

1.1.1 Epidemiology

Cancer places a tremendous burden on society in developing and developed countries equally. The prevalence of cancer is increasing because of the growth and aging of the global population, and an increasing incidence of well-known risk factors, like, for instance, smoking, obesity, and physical inactivity (Ferlay et al., 2015, Tangen, 2016). According to recent statistics, roughly 14 million new cancer cases and 8 million deaths were recorded in 2012 around the world (Torre et al., 2015). Throughout the years, that burden has shifted to developing countries, which as of now have recorded just below 60% of cases and 65% of cancer deaths worldwide (Ferlay et al., 2015, Tangen, 2016). Lung cancer has been found to be the main cause of cancer deaths amongst males, and similarly, breast cancer is the primary form of cancer deaths amongst females in both developed and developing countries (Dela Cruz et al., 2011, Mitchell et al., 2014). Further increases of cancer incidence in developed countries is due to the appearance of colorectal cancer amongst both sexes and prostate cancer in males (Jemal et al., 2011). In developing countries, liver and stomach cancer amongst

males and cervical cancer in females are likewise causing cancer death. Despite the fact that incidence rates for all cancers combined are about twice as high in developed versus developing countries in both sexes, death rates are just 8% to 15% higher in more developed countries (Torre et al., 2015, Jemal et al., 2011). This disparity reveals regional differences in detection practices and/or the accessibility of treatment.

The risk factors for cancer comprise tobacco usage (lung, stomach, liver, and colorectal cancer), obesity and physical inactivity (breast and colorectal cancer) and infection (stomach, liver, and cervical cancer). A major proportion of cancer cases and deaths might possibly be counteracted by extensively applying effective prevention measures, e.g., tobacco control, immunization, and the utilization of early detection tests. Further unexpected deaths could be reduced with appropriate treatments. Much remains unknown of the origins of significant tumours, including pancreatic, prostate, and hematopoietic malignancies. An intensified and concerted effort from all parts of society, such as governments, the public and the private sector, is essential to seize control of the cancer developing burden (Torre et al., 2015).

1.1.2 Metastasis

The process by which cancer cells spread from the primary site to distant organs is known as metastasis. Various radical phenotypic and biochemical modifications occur throughout the transformation of a healthy cell into an invasive cancer cell. These changes involve cell–cell adhesion, growth factor signalling, motility or cell shape and gene expression (Leber and Efferth, 2009). Cancer cells originating from epithelium can lose their usual abilities and

features, adopting a mesenchymal-like phenotype; this is known as an epithelial-mesenchymal transition (EMT) (Jing et al., 2011). The EMT phenomenon takes place in the course of normal embryonic development, and is identified by loss of cell adhesion and acquisition of cell motility. It is reported that EMT is regulated through down-regulation of E-cadherin (an epithelial marker) via transcriptional repressors like Snail and Zeb. Significantly, EMT is also found to be implicated in cell invasion, and formation of side populations of cancer stem cells (Lim et al., 2014).

Many oncogenes, TSGs and metastasis suppressor genes have the ability to influence the invasiveness and the metastatic likelihood of tumour cells (Yokota, 2000, Takaoka et al., 1998). The innate and adaptive immunity cells, adjacent stromal cells and chemokines, as well as their corresponding receptors, play a central role in cancer metastasis (Leber and Efferth, 2009, Jing et al., 2011). Moreover, the microenvironment, vascularisation and support of specific cytokines affect these modifications.

There are three key types of cell involved in tumours - transit amplifying cells, terminally differentiated cells (TD) and CSCs (Alison et al., 2010, Sell, 2010). The latter are believed to have the ability to proliferate indefinitely; hence, they possibly will be the cells that effectively spread and primarily build the majority of the clinically relevant metastases (Leber and Efferth, 2009). There are chains of complex interactions between cancer cells and their microenvironments that are involved in cancer metastasis. These interactions can affect the cell's biological efficiency and enable cancer cells to arrest in distant organs (Peddareddigari et al., 2010, Koontongkaew, 2013).

Most deaths from cancer are mainly because of metastases especially when they become resistant to ordinary treatments. The biological heterogeneity of tumour cells in the primary neoplasm and in metastases is the foremost obstacle to their treatment (Fidler, 2003). Moreover, the particular organ microenvironment can alter the response of a metastatic cancer cell to systemic treatment. Persistent empiricism in the treatment of cancer metastasis is unlikely to generate major developments in cancer therapy (Langley and Fidler, 2007). Thus, it has become crucial for cancer researchers to study the pathogenesis of metastasis at the systemic, cellular, and molecular levels in order to accomplish their goal of curing cancer. The process of cancer metastasis comprises extended chains of consecutive, interrelated steps. Every single step can be rate-limiting, as an inadequacy or a failure at any one step can end the whole process (Fidler, 2002, Fidler, 2003). The effect of the process is reliant on both the fundamental properties of cancer cells as well as the host's response (Figure 1-1).

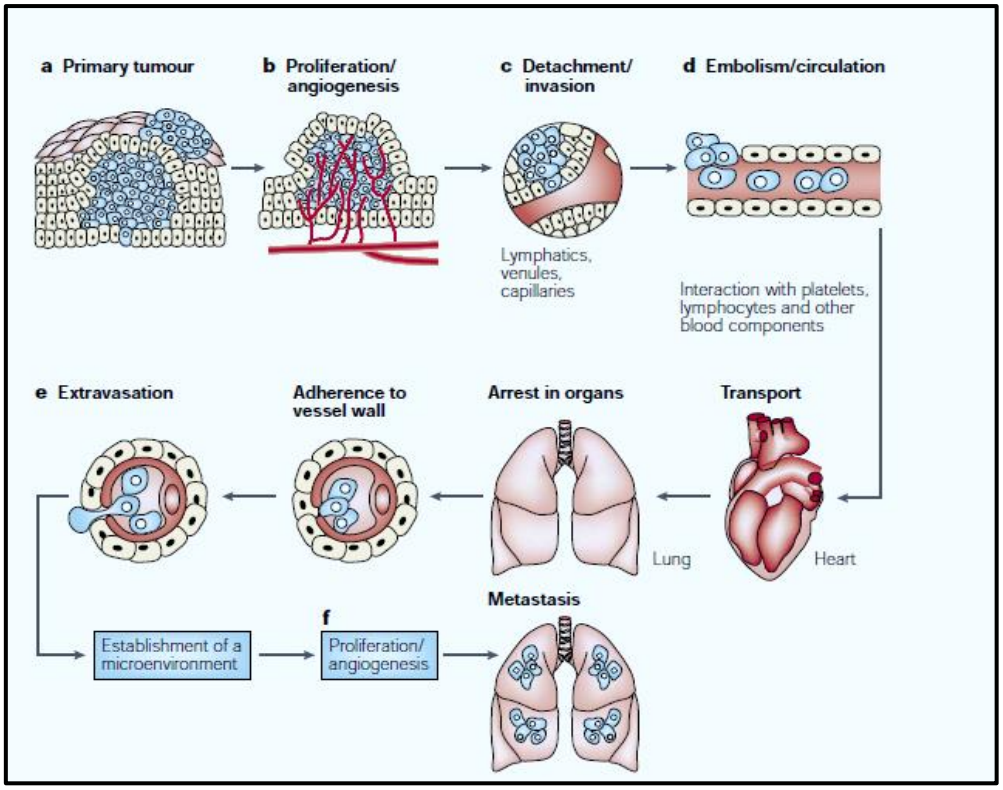


Figure 1-1. The principal steps in the development of cancer metastasis (Fidler, 2003)**. A**. Cellular alteration and tumour development. For the development of malignant tumour cells, there is the necessity for it to be progressive, with supplements for the growing tumour mass at first supplied by straightforward simple diffusion. **B.** Broad vascularisation must happen if a tumour mass is to surpass 1–2 mm in diameter. The production and secretion of angiogenic factors generate a capillary system from the contiguous host tissue. **C**. Local invasion of the host stroma by certain cancer cells takes place by many analogous mechanisms. Narrow venules, like, for example, lymphatic channels, offer almost no resistance to penetration by cancer cells and provide the most widely recognized route to cancer cell entrance into circulation. **D**. Detachment and embolization of definite cancer cells or aggregates take place and, then, the majority of circulating cancer cells are rapidly destroyed. In what follows, the tumour cells that survive circulation wind up in the capillary beds of distant organs by adhering either to capillary endothelial cells or to a sub-endothelial basement membrane that may possibly be exposed. **E.** Extravasation occurs afterward - most likely by mechanisms a kin to those functioning during invasion. **F.** Proliferation within the organ parenchyma ends the metastatic process. To keep developing, the micro-metastasis must build up a vascular network and avoid damage by host defences. The blood vessels can then be invaded by cancer cells that pass in the circulation and produce further metastases**.**

1.1.3 Cancer Stem Cells and Tumour Propagation Models

The process of metastasis is thought to be mediated by "cancer stem cells" (CSCs). This term refers to a subpopulation of the malignant cells that have the capability of self-renewing and giving rise to cells that comprise the tumour (Reya et al., 2001, Clarke et al., 2006). These cells have been dubbed cancer stem cells due to their 'stem-like' characteristics. CSCs share significant features with normal tissue stem cells, encompassing self-renewal (symmetrical and asymmetrical division) and differentiation capacity. However, multilineage differentiation is not an essential feature of a CSC. One apparent implication of the CSC model is that tumours are hierarchically arranged with CSCs lying at the upper part of the hierarchy (Fig. 1) (Visvader and Lindeman, 2008). The original concept of CSCs originated from acute myeloid leukaemia, where a rare subset of cells, amounting to less than or equal to 1% of the total population could initiate leukaemia when transplanted into immunodeficient mice (Lapidot et al., 1994, Visvader and Lindeman, 2008).

The clonal evolution model, in contrast to the CSC model, assumes that mutant malignant cells with a developmental advantage are selected and extended, with each of the cells in the predominant population having equal chance for redeveloping cancer growth (Figure 1-2). The accumulation of genetic events supports this model although epigenetic changes and microenvironmental modifications are additionally liable to have vital roles. The clonal evolution model might, occasionally, include a stochastic component (Visvader and Lindeman, 2008).

Both patterns of tumour propagation are prone to occur in human cancer, but just the CSC model is hierarchical. It is crucial to be cognizant of the fact that the two models are not mutually exclusive, as CSCs themselves experience clonal evolution, just as per what appears in the case of leukaemia stem cells (Barabe et al., 2007). Hence, a second, more overwhelming CSC may develop if a mutation gives more aggressive self-renewal or development properties (Fig. 1-2).

CSCs are different from the cell of origin. The cell of origin particularly alludes to the cell type that obtains the first oncogenic hit(s). Additionally, it is not necessary for CSCs to be initiated from the transformation of normal stem cells. CSCs might emerge from restricted progenitors or further differentiated cells that have obtained the ability of self-renewal. One outcome of this model is that there will be mechanistic similarities between the self-renewal programmes of normal stem cells and CSCs. It has been supposed in several scenarios that the cells in which tumours arise are committed cells that have experienced a certain degree of growth and differentiation (Visvader and Lindeman, 2008).

Metastasis seems to depend on acquisition of EMT and stemness. CD24 confers both of these features; therefore, it is probably worth testing.

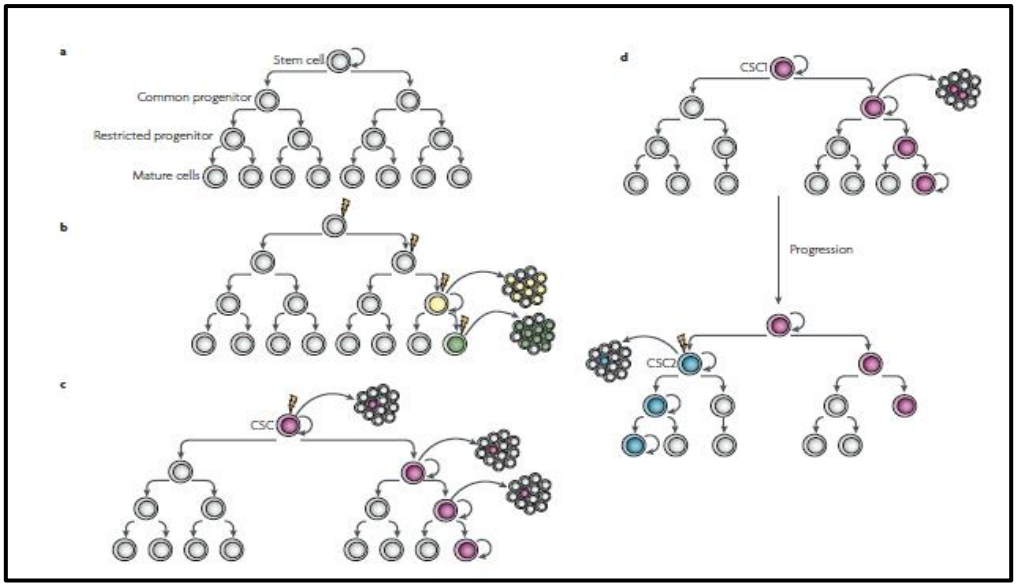


Figure 1-2. Models of cancer heterogeneity and propagation (Visvader and Lindeman, 2008). **A**. Normal cellular hierarchy containing stem cells (at the top), which aggressively produce restricted progenitor or further differentiated cells, eventually responsible for creating all the mature cell types that establish a specific tissue. **B**. In the clonal evolution model, all undifferentiated cells have comparable tumourigenic capacity. **C**. With the CSC model, only the CSC can produce a tumour in view of its self-renewal characteristics and massive proliferative potential. **D**. Both tumour models' upkeep might underlie tumourigenesis. At first, cancer growth will be driven by a particular CSC (CSC1). With tumour development, a further distinct CSC (CSC2) could emerge because of the clonal evolution of CSC1. This is possibly the result of gaining another mutation or epigenetic alteration.

1.2 CD24

1.2.1 CD24 Gene Structure

The cluster of differentiation 24 (CD24) was initially discovered in mice in 1978 as a heat-stable antigen (HSA) (Jaggupilli and Elkord, 2012, Pruszak et al., 2009). Shortly thereafter, the mouse CD24 gene was cloned and acknowledged as a small protein with a mature peptide of 27 amino acids (Kay et al., 1990). A year later, the human CD24 gene was identified (Kay et al., 1991). The human CD24 gene has been described as a single exon gene mapped to chromosome 6q21 as detected by *in situ* hybridization (Ahmed et al., 2009b, Kristiansen et al., 2003b, Hough et al., 1994). It has also been reported to be localised to

chromosomes 1 and 15 along with the Y-chromosome; however, these are all pseudo-genes as they are not functional (Sagiv et al., 2008, Fang et al., 2010). CD24 mRNA is 2179 bp with an open-reading frame of 243 bp (from 111 to 353). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated human CD24 has a varied molecular weight ranging from 20 and 70 kDa, which is probably based on its extensive glycosylation - the protein core forms only 5–10% of the apparent molecular mass (Henniker, 2001, Liu and Zheng, 2007b).

CD24 cDNA has various noteworthy features. First, it has a remarkably long 3' (three prime) untranslated region (UTR). Of the ~2 kb mRNA, slightly over 10% is utilised to encode a small protein, whereas the residual 90% constitutes the 3' UTR. A widespread deletion analysis was carried out by (Zhou et al., 1998), identifying the role the 3' UTR plays in regulating the stability of mice CD24 mRNA. Analyses performed on human CD24 polymorphisms showed a dinucleotide deletion of human CD24 mRNA that significantly reduced the stability of mRNA. Even though significant conservation was discovered between mice and human CD24 mRNA, the predicted sequence of amino acids of mature proteins encoded by mice and human CD24 were found not to be conserved (Kay et al., 1991, Wenger et al., 1991). However, the fundamental feature of a mucin-type structure was observed to be conserved. This conservation indicates that it is possibly the glycosylation pattern, rather than a particular amino acid sequence, that is critical for the function of the CD24 molecule (Liu and Zheng, 2007a).

1.2.2 CD24 Protein Structure

CD24 is a small, heavily glycosylated cell-surface protein, attached to cell membranes by a glycosyl phosphatidylinositol (GPI) anchor (Kristiansen et al., 2004a, Jaggupilli and Elkord, 2012). The CD24 mature protein is approximately 27 amino acids long in mice and 31 amino acids in humans (Schabath et al., 2006, Bretz et al., 2012a), and most of the protein molecular mass contains extensive N- and O-linked glycosylation sites. Human CD24 has additional serine and threonine residues, rendering the molecule like-mucin (Figure 1-3) (Baumann et al., 2005, Kristiansen et al., 2004a).

CD24 protein consists of an N-terminal signal peptide, which allows it to be localised onto the cell surface, and a C-terminal region (GPI-displaced tail), which suggests a pro-peptide that at the binding site of the GPI anchor in the C-terminal region of the CD24 mature peptide, thereafter being cleaved (Kristiansen et al., 2003c).

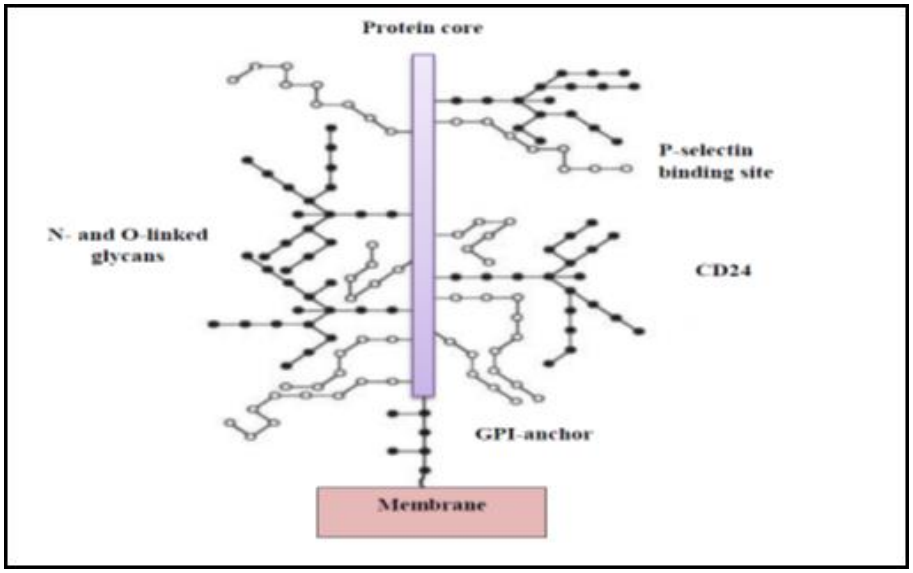


Figure 1-3. The schematic model of human CD24 (Kristiansen et al., 2004a). The gene is composed of a small protein core of 31 amino acids attached to the cellular membrane through a GPI anchor. This protein core has several glycosylation sites for O-linked carbohydrates, rendering the molecule like- mucins.

1.2.3 Function of CD24

1.2.3.1 CD24 Expression in Normal Tissues

CD24 is typically expressed in haematopoietic system cells (e.g., B-cell precursors and neutrophils), neuronal tissue and a number of epithelial cells (e.g., renal tubular epithelium and keratinocytes) (Sagiv et al., 2008). A previously published study revealed that CD24 is commonly used as a marker for the differentiation of various lineages of cells and can act as a co-stimulator for T-cells, mainly in non-lymphoid target organs. CD24 had also been stated to bind to a member of the immunoglobulin superfamily, L1, expressed on both neural and lymphoid cells (Baumann et al., 2005). According to Kadmon et al., CD24 acts to form a functional complex of neuronal adhesion molecules and it could induce the signal transduction pathway of L1, possibly in response to neuron-neuron contact (Kadmon et al., 1995). Furthermore, CD24 tends to be more highly expressed in progenitor cells versus differentiated cells. Therefore, the necessity for more studies to elucidate its mechanisms in both differentiated and undifferentiated cells has become critical (Jaggupilli and Elkord, 2012).

In our group, CD24 expression was immunohistochemically evaluated in a large series of CRC tissues and 10 whole-tissue sections of both tumour and normal mucosa (Ahmed et al., 2009a). Both luminal membranous and cytoplasmic CD24 expressions were detected in CRC cases, while no membranous CD24 expression was observed in normal epithelium, but CD24 expression was limited to the base of the crypts (i.e. where the stem cells are) (Figure 1-4).

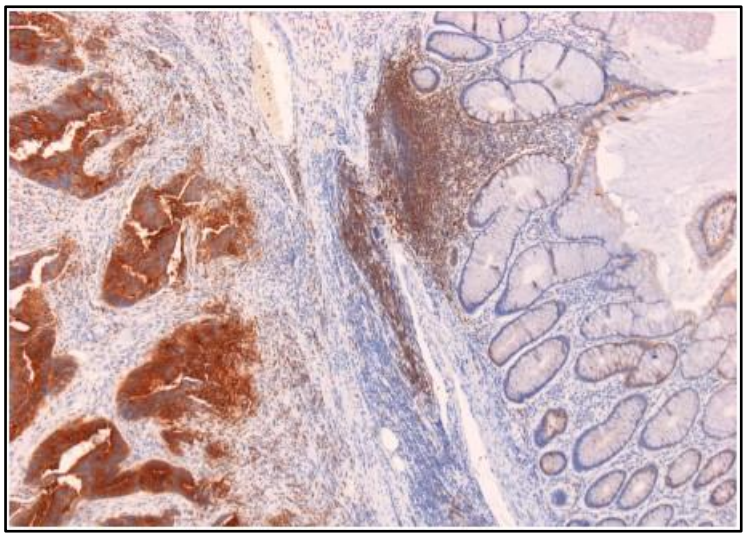


Figure 1-4. Patterns of CD24 immunostaining in normal colonic tissue. A weak CD24 expression in the base of crypts (Ahmed et al., 2009a).

1.2.3.2 The Role of CD24 in Inflammation

CD24 has been reported to play a crucial role in genetic checkpoints for various autoimmune diseases in both humans and mice (Liu and Zheng, 2007a, Wang et al., 2007c). For instance, its activity as a genetic modifier has been established within cases of multiple sclerosis and systemic lupus erythromatosis (Wang et al., 2007b, Zhou et al., 2003, Liu and Zheng, 2007b). CD24 has also been documented to play a role in inflammation as a consequence of its binding with a number of damage-associated molecular patterns (DAMPs), such as high mobility group box protein 1, members of the heat shock protein (HSP) family and nucleolins (Chen et al., 2011), and similarly because of its ability to suppress the host response to tissue injury through its contact with Siglec-10 in dendritic cells (Chen et al., 2009). Recently, CD24 has been characterised as a glycosylated protein marker of intestinal crypt stem cells, and Paneth cells were observed to be up-regulated in inflammatory bowel disease (IBD) (Sato et al., 2011). Even more recently, Parlato and colleagues (Parlato et al., 2014)

evaluated the likelihood of involving CD24 in the modulation of neutrophil function. The results illustrated that the expression of CD24 was altered by pro- and anti-inflammatory mediators, and that CD24 cross-ligation can significantly accelerate neutrophil death in a caspase-dependent fashion. A further finding was that the expression of CD24 is down-regulated in neutrophils from patients with sepsis, and is associated with an impaired CD24-mediated cytotoxic response and neutrophil death. Taken together, these results propose novel aspects of CD24-mediated immunoregulation, providing evidence for its potential role in the pathophysiology of sepsis.

1.2.3.3 CD24 Expression in Cancer

CD24 is up-regulated in several types of tumours, including breast cancer, hepatocellular carcinoma, pancreatic adenocarcinoma, small cell lung cancer, prostate carcinoma, ovarian cancer, CRC and cholangiocarcinoma (Kristiansen et al., 2004a, Kristiansen et al., 2003b, Lee et al., 2009b, Weichert et al., 2005b). In certain tumours, the overexpression of CD24 has been determined to be related to poor clinical outcomes (Kristiansen et al., 2003c, Kristiansen et al., 2004a, Agrawal et al., 2007). With this, CD24 has been stated to function as a regulator of proliferation, invasion, metastatic tumour spread and apoptosis in cancer (Su et al., 2012). Additionally, it has been seen to play a fundamental role in cell-cell and cell-matrix interactions (Lee et al., 2010, Kristiansen et al., 2003b). It promotes the processes of adhesion of tumour cells to collagen, laminin and fibronectin, as well as proliferation (Jaggupilli and Elkord, 2012). Recent studies have demonstrated that CD24 coexists with other CSC markers - such as CD29, CD31 and CD44 - in different tumours (Jaggupilli and Elkord, 2012, Szafarowski and Szczepanski, 2014, Hofner et al., 2014). Mierke et al.,

(Mierke, 2011) demonstrated that CD24 behaves as a regulator for β1 integrins located on the cell surface. Although CD24 is localised in lipid rafts, its function in regulating β1 integrins and the entry of chemokine receptor type 4 (CXCR4) into this specific membrane domain remains unclear.

1.2.3.4 The Role of CD24 in Stimulating Cell Motility, Invasion, and Stemness

A large and growing body of literature has suggested the involvement of CD24 in tumour cell motility and invasion (Baumann et al., 2005, Taniuchi et al., 2011, Mierke, 2011). For instance, in breast cancer, MCF-7 cells with a high expression of CD24 were seen to have greater migration and invasion abilities (Kim et al., 2007). In CRC, forced expression of CD24 increased cell migration, invasion and colony forming efficiency (all $p < 0.05$), while it did not affect cell proliferation and apoptosis. Equally, down-regulation of CD24 reduced cell migration and invasion (all $p < 0.05$), while it did not affect cell proliferation, and apoptosis or staurosporine-induced apoptosis (Ahmed et al., 2010). These data were generally consistent with those that have been shown in an earlier study (Sagiv et al., 2008). A recent study reported that CD24 could regulate the EMT and stemness through Notch1 signalling in breast cancer cells (Lim et al., 2014).

A meta-analysis to investigate CD24 expression in cancer was carried out by (Lee et al., 2009a) using immunohistochemistry (IHC). It was reported that the CD24 expression frequency was nearly 70% in all studied carcinomas, including breast, GIT, prostate, pancreas, skin, female reproductive and urinary system. CD24 was also described as being often overexpressed in carcinomas compared

to benign lesions, and that it is considerably aligned with lymph node metastasis, progressive clinical stages and reduced overall survival.

As such, this meta-analysis strongly reinforced the notion that CD24 is a crucial marker of malignancy and poor prognosis in several tumours.

Other investigations performed on CD24, including using flow cytometry and mRNA real-time PCR, showed that the expression of CD24 was highly down-regulated in invasive tumour cell lines (Schindelmann et al., 2002). Nevertheless, the locally aggressive behaviour of CD24-positive gliomas in a mouse model were described, suggesting an inconsistency with the earlier literature (Lim, 2005).

1.2.3.5 The Role of CD24 in Stimulating Metastasis and EMT

It has been proven that CD24 acts as a ligand of P-selectin (i.e., an adhesive molecule on activated endothelial cells and platelets) (Aigner et al., 1997). Such binding is believed to aid in the dissemination of tumour cells and it could be a vital factor in recruiting leukocytes to the targeted cells and infected tissues. Furthermore, as a result of the interaction between CD24 and P-selectin, and considering that P-selectin is expressed in blood vascular endothelial cells and lymphatic vascular endothelial cells (Friederichs et al., 2000), this might be a novel mechanism of CD24-mediated metastasis. Of late, CD24 has been described as a putative stem cell marker, meaning that it might have various functions that are especially associated with cancer biology, though this is debated and still being intensely investigated (Ahmed et al., 2009a). The mechanisms by which CD24 can regulate cellular functions in cancer, overall, continue to be unveiled (Bretz et al., 2012a). CD24 seems to regulate the EMT

markers through targeting Src, required for the activation of FAK that has been reported to induce cell activity to stimulate EMT markers (Cicchini et al., 2008).

1.2.4 The Main Signalling Pathways Associated with CD24 Expression

CD24 may achieve cell functional activity through various signalling pathways including its upstream regulators, downstream targets and other CD24-related pathways. The following important pathways may be involved in this process will be highlighted in more detail.

1.2.4.1 c-Src Pathway

The localisation of CD24 within glycolipid-enriched membrane (GEM) domains provides it a specific location in the plasma membrane, where most of the cellular signalling occurs (Zarn et al., 1996, Baumann et al., 2012). Within the lipid rafts in breast cancer cells, CD24 has been found to interact with c-Src, accordingly increasing its activity (Baumann et al., 2012). There, Src family kinases (SFKs) are located upstream of the mitogen-activated protein kinases (MAPK) cascades in multiple receptor signalling systems (Pazdrak et al., 1995). Src family kinases are found abundantly within mammals and are a member of a family of non-receptor-type tyrosine kinases (Bjorge et al., 2000, Yeatman, 2004). One example within this family is Lyn, which is highly expressed in myeloid cells and B-lymphocytes. Lyn was observed to be able to serve as a positive as well as negative modulator of many signalling responses (Janas et al., 1999). An association between Lyn kinase in erythroleukemia cell lines and the activity of CD24 has also been acknowledged.

The activity of Lyn had been reported to increase in multiple human tumours. Its activation commonly takes place in breast cancer, glioblastoma and prostate

cancer along with CRC (Choi et al., 2010, Montero et al., 2011, Hao et al., 2010). Consequently, it was hypothesised that CD24-induced extracellular signal-regulated kinase 1/2 (ERK1/2) activation involved SFKs. Recently, Su et al. investigated the relationship between Lyn activity and CD24 within CRC. The results uncovered that CD24 and Lyn interacted. It was also seen that CD24 was responsible for the induction of the activation and nuclear translocation of Lyn. In contrast, when Lyn was inactivated, CD24-induced cell invasion and ERK1/2 activation were inhibited in CRC cells. IHC experiments carried out on CRC tissues demonstrated that the Lyn and CD24 expression were correlated with the development of tumours and the progression of metastases. The study concluded that CD24 expression is correlated with the activation of the ERK1/2 and Lyn, and might therefore be involved in the mechanism related to CD24-mediated regulation of CRC progression (Su et al., 2012).

c-Src plays a vital role in inducing cancer invasion and metastasis (Brunton and Frame, 2008, Baumann et al., 2012). As reported in a recent study by Baumann and colleagues (Baumann et al., 2012) on MTLy breast cancer cells, CD24 collaborates with and enhances the kinase activity of the c-Src. This occurred within and was reliant on the intact lipid rafts. The researchers also found that the CD24-augmented c-Src kinase activity induced the formation of focal adhesion complexes and increased integrin-mediated adhesion, leading to acceleration of the phosphorylation of the focal adhesion kinase (FAK) and Paxillin. The gain and loss of function approaches exhibited that c-Src activity was necessary for mediating the effects of CD24 on integrin-dependent adhesion and cell dissemination in addition to invasion. These outcomes suggest that c-Src is a CD24-activated mediator which underpins integrin-mediated adhesion

and invasion. They also imply that CD24 contributes to the progression of a tumour by stimulating the activity of c-Src or other members of the Src family. In keeping with this, several cell lines from colon and breast cancer have been thoroughly studied (Asangani et al., 2008) and Src was found to be activated by CD24, which also contributes to induction and activation of c-Jun and c-Fos. Consequently, promoter activity and both RNA and microRNA-21 (miR-21) expression are increased by CD24. The latter suppresses the expression of programmed cell death 4 (PDCD4) as well as that of phosphatase and tensin homolog (PTEN). Co-transfection of a CD24 expression construct and silencing Src by small interfering RNA (siRNA) has shown that Src mediated the CD24-dependent up-regulation of miR-21. It was also discerned that miR-34a post-transcriptionally down-regulated the expression of CD24 and Src. This resulted in the deactivation of c-Jun, decreased expression of c-Jun and c-Fos, up-regulated PDCD4 and PTEN and inhibited miR-21(Meng et al., 2007, Asangani et al., 2008).

A decline in the migration and invasion of CRC cells was also detected because of miR-34a-mediated inhibition of Src expression (Muppala et al., 2013). This relationship was confirmed in tumour tissues that were obtained from patients with CRC which exhibited a manifest decrease in the expression of PDCD4 and miR-34a and an elevation in the expression of CD24, Src and miR-21 in comparison with the analogous normal tissues. Additionally, there was a direct relationship between CD24 and the Src protein in tumour tissues and an inverse correlation between miR-34a and Src protein levels.

1.2.4.2 PI3K/AKT Pathway

The phosphoinositide-3 kinase (PI3K)-AKT pathway has been reported to be involved in the molecular pathogenesis of different malignancies. Its overexpression was implicated in the initial stages of colorectal carcinogenesis (Johnson et al., 2010). The activation of the AKT pathway has been found to be influenced by CD24, specifically by enhancing phosphorylation at the S473 site (AHMED, 2011). AKT is fully activated through the independent activation of two phosphorylation sites, S473 and T308 (Qiao et al., 2008). Moreover, 3-phosphoinositide-dependent protein kinase-1 (PDK1) was described to phosphorylate AKT on Thr308, which is entirely dependent on PI3Kinase (Vanhaesebroeck and Alessi, 2000a). The phosphorylation at Ser473, on the other hand, is prompted by mammalian target of rapamycin complex 2 (mTORC2) (Vivanco and Sawyers, 2002). As human epidermal growth factor receptor 2 (HER2)-positive breast tumours are nearly 100% positive for CD24, Hosonaga et al. (Hosonaga et al., 2014) suggested that such an association between the expression of both molecules is more than likely to exist. The expression of CD24 was seen to be augmented by overexpression of HER2 in certain breast cancer-negative cell lines. Moreover, knockdown of CD24 in breast cancer higher expressor cell lines of endogenous HER2 resulted in a down-regulation of HER2 expression, while the expression of CD24 was not affected by knockdown of HER2. It has also been noted that the expression of AKT-phosphorylation (downstream of HER2 and PI3K) is suppressed following knockdown of CD24. Altogether, these findings suggest that CD24 enhances the expression of HER2 and the activation of PI3K-Akt signalling. Broadly speaking, such an association between CD24 signalling and PI3Kinase

signalling is indicative of convergence upon the activation of AKT and downstream effectors, eventually leading to the enhancement of tumourigenic and metastatic features of cancer cells.

1.2.4.3 Signal Transducer and Activator of Transcription 3 (STAT3) Pathway

Human cancer cells were used as a model to identify how CD24 affects cellular functions (Bretz et al., 2012b). It was observed that the expression of STAT3-dependent genes was altered by the knockdown or overexpression of CD24; FAK and STAT3 phosphorylation, as well as STAT3-transcriptional activities, were affected by CD24 depletion; Src activity was essential for the CD24-mediated regulation of STAT3 genes; and Src activity and STAT3 gene expression *in vivo* were altered by the treatment of mice bearing A549 or BxPC3 tumours with mAbs directed against CD24. The model illustrated in Figure 1-5 summarises CD24's role in the regulation of STAT3 and FAK activities through Src, supporting its relationship with a poor prognosis in cancer.

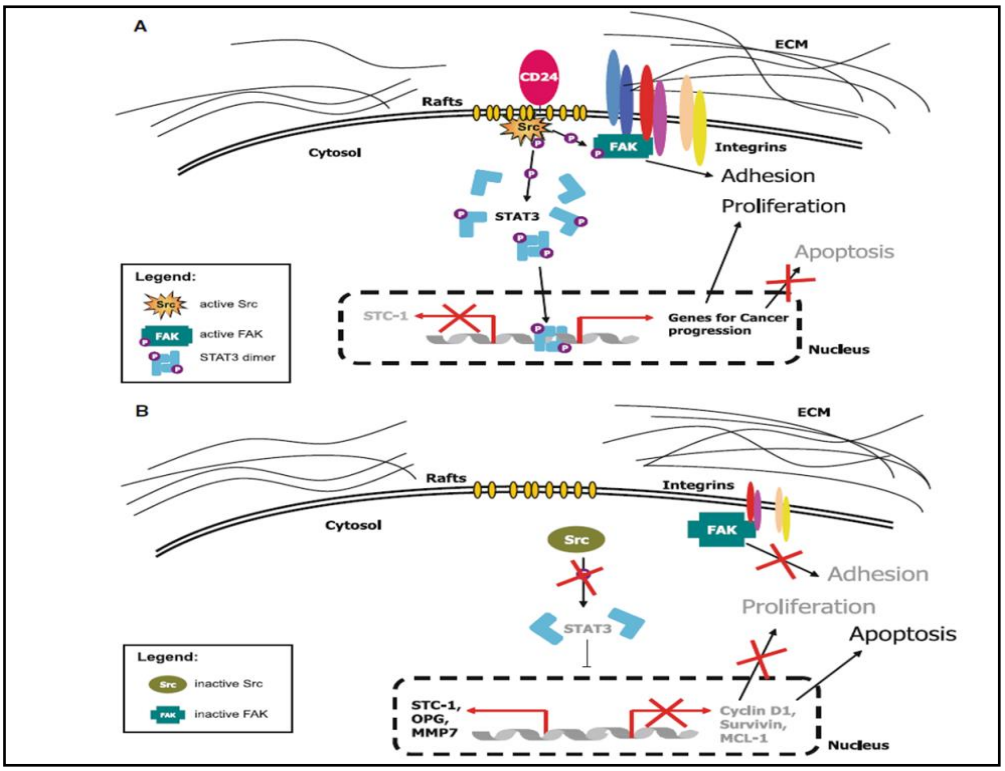


Figure 1-5. CD24 role in the regulation of STAT3 (Bretz et al., 2012b). **A.** The localisation and the activity of Src kinase in lipid rafts are reinforced by the existence of CD24. Src activates the STAT3 molecules via phosphorylation at tyrosine 705. The activated STAT3 is dimerised and translocated into the nucleus. Next, the DNA binding of the STAT3 dimers lead to the expression of specific target genes that might alter cell characteristics, such as survival and proliferation, encouraging oncogenicity of the tumour cell. The activation of FAK might also influence integrin-dependent cell adhesion to ECM substrates. **B.** The displacement of Src kinase from the rafts and the weakening of its activity occurs because of the silencing of CD24 and the consequent depletion of the lipid rafts. A decrease in the activation of the STAT3 resulted in the reduction of STAT3 target gene expression, affecting the proliferation of tumour cells and promoting apoptosis. Impairment of FAK-regulated adhesion to the ECM also took place.

1.2.4.4 ERK/MAPK Pathway

Signal transduction from the cellular surface to the nucleus is achieved by MAPKs, which are serine/threonine kinases. MAPK in mammals can be categorized into c-Jun N-terminal kinase (JNK), ERK, and p38 MAPK. MAPK cascades, in response to a variety of extracellular stimuli, can dictate the fate of cells, including cellular growth, differentiation and apoptosis (Gaestel and Kracht, 2009). One study illustrated that there was an inverse relationship

between CD24 expression and the phosphorylated mitogen-activated protein kinase (p-MAPK) in cholangiocarcinoma, demonstrating that MAPK pathways could be involved in CD24-induced tumourigenesis (Agrawal et al., 2007). Another study investigated the effect of CD24 on the proliferation of CRC cells and attempted to illuminate the likely role of the MAPK pathway in this process. The findings alluded to CD24-dependent ERK1⁄2 and p38 MAPK activation being essential for CRC cell proliferation both *in vivo* and *in vitro*. The study also suggested that the relationship between CD24 and MAPK pathways perhaps shed light on a novel mechanism in the regulation of CRC proliferation (Wang et al., 2010).

1.2.4.5 Wnt Pathway

The CD24 gene has been reported to be directly reactive to the *Wnt* signalling pathway, and consequently might be involved in stem cell maintenance (Yang et al., 2009). For instance, Ahmed and colleagues reported that the existence of the T-cell factor/lymphoid enhancer factor (TCF/LEF) consensus sequences in the promoter region of CD24 had made it a likely target gene of the canonical *Wnt* signalling pathway. In addition, a direct association between β-catenin and CD24 has been confirmed. Specifically, the activity of β-catenin was first inhibited using a vector expressing dominant-negative TCF4 (DN-TCF4) in a colorectal cell line (HT29). After the transfection, CD24 expression levels were assessed. It was observed that the forced expression of the DN-TCF4 protein led to the down-regulation of CD24 mRNA and CD24 protein, providing evidence that the CD24 gene was a target of the *Wnt* signalling in colon cancers (Ahmed et al., 2010).

In breast cancer, it was found that the accumulation of β-catenin in the cell cytoplasm and in the nuclei, an output of *Wnt* pathway activation, was the most common observation in the basal-like subtype of *in situ* and invasive cancers. It was also observed that in cases of *in situ* and invasive tumours, both cytosolic and nuclear β-catenin were correlated with other characteristics of basal-like tumours, like oestrogen receptor (ER) and progesterone receptor (PR) negativity, as well as EGFR, cytokeratin 5/6 (CK 5/6) and vimentin expression. In invasive tumours (not *in situ* tumours), the accumulation of β-catenin had been found to be linked to the $CD44^{+}/CD24^{-}$ profile. Based on this, the activation of the *Wnt* pathway in these types of tumours could be an indication of their enriched stem cell composition (Khramtsov et al., 2010).

1.2.4.6 GTPase Pathway

A previously published study (Smith et al., 2006) identified the metastasis-associated protein CD24, a downstream target of Ral signalling, through profiling the expression of Ras-related protein Ral-A (RalA) and Ras-related protein Ral-B (RalB)–depleted bladder carcinoma cells. The loss of CD24's function in the cell lines obtained from various common cancer types was uncovered to be an indicator of a reduction in cellular proliferation, clonogenicity in soft agar, actin cytoskeleton modifications and stimulation of apoptosis. Taking these phenotypes into account, a human cancer bladder tissue microarray was evaluated using the IHC technique for CD24 to identify whether CD24 is a prognostic cancer biomarker. It has been found that an increase in CD24 expression was associated with shorter patient disease-free survival. Hence, CD24 is a functionally significant Ral-regulated target and a possibly crucial cancer prognostic marker. Neel et al., (Neel et al., 2011), in another

study, found that the suppression of CD24 expression in the urothelial bladder cancer cell line, UMUC-3, as well as in other cell lines, shortened anchorage-independent proliferation and survival, indicating that CD24 up-regulation might be a key element of Ral-mediated oncogenesis.

1.2.4.7 Tissue Factor Pathway Inhibitor 2 (TFPI-2)

The depletion of CD24 reduces the invasion of tumour cells and up-regulates TFPI-2, a strong inhibitor of ECM degradation that has the ability to block the formation of metastasis and the invasion of cancer cells (Su et al., 2012, Bretz et al., 2012a). It was identified that the overexpression of CD24 in A125 cells in breast cancer leads to a decrease in TFPI-2 expression and to more invasion. It was also stated that CD24 knockdown affects c-Src activity. The splicing of the c-Src and CD24 was observed to increase TFPI-2 expression and reduce the invasion of tumour cells. IHC analyses of primary breast cancer cells revealed an inverse correlation between the expression of CD24 and TFPI-2. The CD24 negative samples exhibited hyper-expression of TFPI-2, but that expression was reduced with the rise of CD24 expression. There is evidence that patients with a CD24 low/TFPI-2 high phenotype demonstrate pronouncedly enhanced survival versus CD24 high/TFPI-2 low patients. Consequently, CD24 may regulate cellular invasion through TFPI-2 and there is a likelihood c-Src is part of this (Bretz et al., 2012a).

1.2.5 CD24 Therapeutic Applications

It has been proposed that CD24 could play a vital role in the whole GIT and that monoclonal antibodies (mAbs) could be a new therapy modality (Shapira et al., 2011, Sagiv et al., 2008). As a treatment, mAb is used with a limited quantity of genes. Several of these genes overlap with genes that have an altered degree of expression following expression of siRNA. It was previously shown that anti-CD24 mAbs do not only prevent aggregation, but they also promote growth inhibition in malignant lymphoma cells that express CD24 through the GEM–dependent mechanism, fostered by the cross-linking with B-cell receptors. In addition, anti-CD24 antibodies have been observed to diminish tumour burden *in vivo* in CRC. Treatment effectiveness is probably associated with the reduction of the accumulation of membrane CD24, which could also be consistent with the long-term effects documented with siRNA (Sagiv et al., 2008). It was also demonstrated that the development of CD24-bearing CRC cells is reduced with exposure to anti-CD24 mAbs (celecoxib); hence, CD24 could be a promising novel therapeutic agent for the prevention and treatment of CRC (Sagiv et al., 2006). Shapira et al., (Shapira et al., 2011) examined the likelihood of anti-CD24 SWA11 mAb would serve as an efficacious cytotoxic agent in CRC cells. The SWA11 mAb was conjugated to a *Pseudomonas* exotoxin derivative (PE38) through an Fc-binding ZZ domain from *Staphylococcal* protein A to produce the immunotoxin, SWA11-ZZ-PE38. The study confirmed that the anti-CD24 SWA11 mAb could carry a PE exotoxin derivative to the CRC cells, causing them to undergo apoptosis without causing toxicity to surrounding normal tissues. This immunotoxin was proposed for further development as novel drug for patients suffering from CRC.

1.2.6 The Relationship between CD24 and Focal Adhesion Assembly

Since CD24 was shown found in complex with integrins at focal adhesions (Runz et al., 2008, Baumann et al., 2012); therefore, it may interact with some focal adhesion-associated molecules to regulate cell functions. More details about FAs and other complexes assembled at FAs that may play a significant role in regulating cell behaviour will be given below.

In the course of stable cell adhesion, integrins cluster with various further proteins and form FAs, which links to the extracellular matrix (ECM) components on its extracellular region, and establishes the location of actin cytoskeletons on the cytoplasmic side of the membrane, modulating various intracellular signalling pathways (Brakebusch and Fassler, 2003). Based on earlier biochemical and cell culture studies, integrins are linked to actin cytoskeletons through numerous molecular linkages and mechanisms, including FA assemblages (Figure 1-6) (Brakebusch and Fassler, 2003, Lo, 2006). In cancer, abnormal expression and altered functioning of FA molecules cause adverse tumour behaviour. Moreover, these proteins apparently do not function independently, but rather work together during tumour growth and cancer metastasis (Yam et al., 2009a).

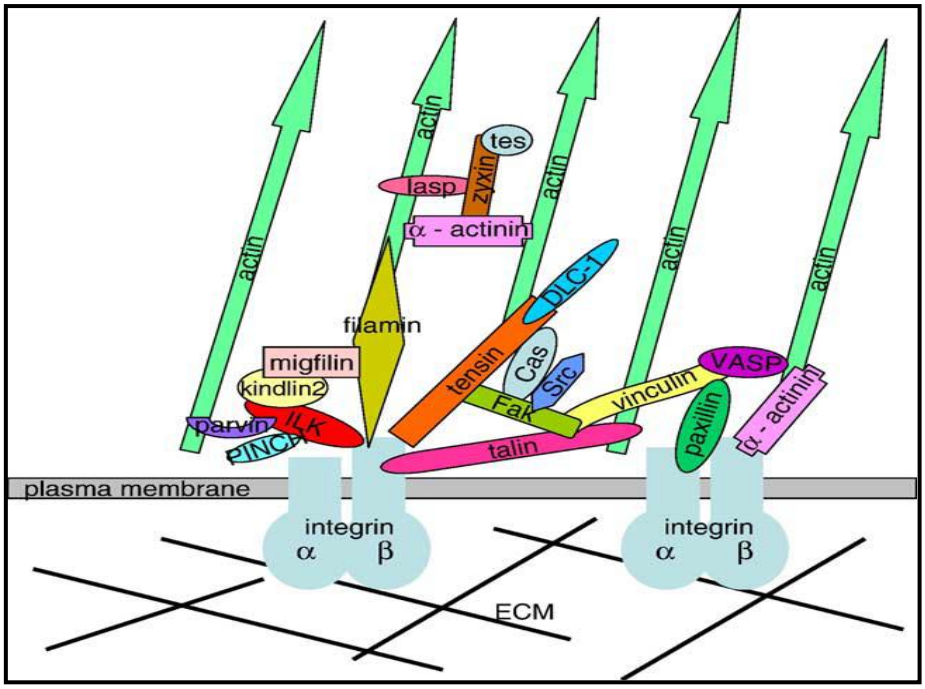


Figure 1-6. FAs assemblages (Lo, 2006). A schematic model showing the interactions between certain components of FAs linking integrin receptors to the actin cytoskeleton.

Several studies have revealed that a diversity of genes are involved in the regulation of FAs. One set of genes that has been recently acknowledged to have a potential role in mediating FAs is the tensin gene family, which probably plays a part in the tumourigenesis process by signalling through certain molecules located in FAs, for instance, FAK and integrin-linked kinase (ILK), or even through other molecules binding to the cytoplasmic tail of integrins (Yam et al., 2009b). Further information about these molecules is included below.

1.2.6.1 The Tensin Family

Tensins are intracellular proteins localised on the cytoplasmic side of FAs, and have become known as novel regulators of cell growth and motility (Yam et al., 2009a, Chen et al., 2002). In 1991, tensin was initially isolated from chicken cardiac muscle. Not long afterwards, human tensin2 was characterised as having

robust homology with tensin at both the C and T termini (Lo, 2004). The tensin gene family is composed of four members: tensin 1 (TNS-1), tensin 2 (TNS-2), tensin 3 (TNS-3) and tensin 4 (TNS-4 or Cten) (Lo and Bin Lo, 2002).

1.2.6.1.1 TNS-4 (Cten)

The C-terminal tensin-like (Cten) is a small protein (21 kb size) that maps to chromosome 17q21.2 (Lo and Bin Lo, 2002). Cten is localised at FAs, and recently, it has also been observed to be localised to the nucleus, yet its significance in this localisation was not defined (Liao et al., 2009). Nuclear Cten expression was described as being associated with advanced tumour stage and metastasis. This indicates that there is a role for Cten beyond cellular adhesion foci (Albasri et al., 2009). The molecular weight of human Cten was reported to be 90 kDa when analysed with SDS-PAGE even though the predicted weight was 77 kDa. Cten has been reported to be widely expressed in many human tumour tissues, while its expression is negligible under normal conditions (Lo and Lo, 2002). Additionally, Cten was found to feature prominently in a number of tumours, and previous studies have demonstrated that Cten activity might vary in tumours of different origins. Cten has been categorised as a tumour suppressor gene in prostate cancer, and interestingly, it has been identified as an oncogene in thymomas, gastric, lung and breast carcinomas (Lo and Lo, 2002, Sasaki et al., 2003b, Sasaki et al., 2003a, Katz et al., 2007). Nevertheless, the role of Cten as an oncogene is exceedingly unclear and requires further study.

1.2.6.2 Focal Adhesion Kinase (FAK)

FAK is a non-receptor tyrosine kinase that resides in FAs complexes and is a central constituent of integrin-mediated signalling (Crossland et al., 2013). CD24 was also reported to have the capability of recruiting integrins to the lipid raft domain and controlling the activity of integrins and other receptor proteins (Baumann et al., 2005, Runz et al., 2008). These types of integrins commence intracellular signal transduction, which is aligned with cancer cell migration, proliferation, and survival. This is in view of the fact that integrins do not have intrinsic catalytic activity - they need to transduce the intracellular signals with the help of adaptor proteins, like Src and FAK. (Mitra and Schlaepfer, 2006). Src phosphorylated by Y416 consecutively phosphorylates Y925 of FAK. This switch leads to the activation of the MAPK cascade (Mitra and Schlaepfer, 2006). In breast cancer cells, it was shown that the cell proliferation and regulation of the cell cycle took place as a consequence of the activation of Src and FAK. In addition, FAK has been found to contribute to transforming growth factor-β1 (TGF-β1)-dependent EMT, as well to the invasion of several breast and renal tubular cell lines. In the process of EMT, FAK aids in the delocalisation of E-cadherin. It also regulates the expression of various EMT marker proteins (Lee et al., 2012).

It has been suggested that E-cadherin might be involved in the process of driving Cten motility into the nucleus. Furthermore, it was found that Cten overexpression led to a decrease in E-cadherin levels, and that a part of Cten's involvement might be based on detaching β-catenin from cadherin junctions and deliver it into the nucleus. Although Cten and FAK localise closely at FAs, the relationship between them is still ambiguous. Based on the data on the role of

Cten in cell migration, such a relationship between Cten and FAK is more likely to exist as both molecules were described to down-regulate E-cadherin (Albasri et al., 2009, Serrels et al., 2011). Modulating Cten expression by either forcibly expressing or knocking down Cten in pancreatic cancer cell lines led to an up-regulation or down regulation of FAK, respectively (Al-Ghamdi et al., 2013). FAK can also bind the Src homology 2 (SH2) domains and co-immunoprecipitate with tensin 1, and it may even perhaps be possible that this molecule directly binds Cten (Yamashita et al., 2004, McLean et al., 2000). Furthermore, a direct relationship between the expression of Cten and of p-FAK was reported, this indicates that ten could be involved in promoting the activation of FAK as well as its expression (Albasri et al., 2014).

1.2.6.3 Integrin-Linked Kinase (ILK)

Another prominent component of FAs is ILK protein, a potential partner of CD24. ILK is a multi-functional molecule that is connected directly to the cytoplasmic domains of β1- and β3-integrins, and might correlate indirectly with actin through other molecules it binds, including Parvin, Paxillin or particularly interesting Cys-His-rich protein (PINCH). Additionally, ILK is described to regulate actin and microtubule dynamics and mechanics through suppressing RhoA-induced actomyosin contractility and stabilizing microtubule tips at nascent FAs (Elad et al., 2013). Other important proteins such as Vinculin and Talin have been reported to interact with some focal adhesion-associated molecules such as FAK or considered as actin binding proteins (Quadri, 2012).

1.2.7 The Significant Role of CD24 in Cancer Development

Several studies have proven that CD24 is implicated in the metastasis and development of several cancer types including CRC (Yeung et al., 2010), pancreas (Sagiv et al., 2006), hepatocellular carcinoma (Lee et al., 2011), and non-small-cell lung cancer (Karimi-Busheri et al., 2013, Lee et al., 2010, Lu et al., 2008). These cancer models share some common features including the embryonic endodermal origin and having frequent mutation of *TP53*, *SMAD4*, and *KRAS* (Hahn et al., 1996, Dergham et al., 1997). Since the main purpose of this study is to investigate the signalling and function activity of CD24 in above cancer models (i.e., CRC, pancreatic cancer, liver cancer, and lung cancer), a brief overview about each cancer type will be included.

1.2.8 Colorectal Cancer

1.2.8.1 An Overview

Colorectal cancer (CRC) is the most significant neoplasm affecting the gastrointestinal tract (GIT) (Duffy et al., 2007, Manxhuka-Kerliu et al., 2009, Dheer, 2015). Initially, it develops from the large intestine, particularly from the colonic and rectal mucosa, through a series of genetic and epigenetic alterations that convert the normal epithelium to adenocarcinomas (Armaghany et al., 2012). The majority of CRCs emerge from sporadic adenomas, while a limited number result from genetic polyposis syndromes or other inflammatory bowel diseases (Fearon, 2011). Almost two-thirds of CRCs are found in the colon, while the remaining cases are observed in the rectum (Cooper et al., 2010). The overall number of cases of CRC has increased because of an aging population

and population growth in both developed and developing countries (Jemal et al., 2011, Etzioni et al., 2009).

Familial adenomatous polyposis (FAP), brought about by a germline mutation of the adenomatous polyposis coli (APC), and hereditary non-polyposis colorectal cancer (HNPCC), which emanates from a mutation in the DNA mismatch repair (MMR) gene, are the most commonly inherited CRCs (Narayan and Roy, 2003). However, other genetic mutations are involved in tumour progression, including tumour protein 53 (*TP53*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), Mothers against decapentaplegic homolog 2/4 (*SMAD2/4)* and deletion of chromosome 18q (Worthley and Leggett, 2010). Recent studies have suggested that the cancer progression is influenced by CSCs, which are not only significant in establishing the primary tumour but also commonly contribute to tumour metastases and recurrences (Di Franco et al., 2013).

1.2.8.2 Epidemiology

CRC is one of the chief causes of morbidity and mortality worldwide - it represents nearly 10% of all cancers (Herszenyi and Tulassay, 2010). Globally, it is ranked as the third most prevalent cancer and fourth in terms of number of deaths (Haggar and Boushey, 2009, Deschoolmeester et al., 2010). Annually, there are approximately one million new cases of CRC, and the total deaths associated are roughly half of this number (Bingham and Riboli, 2004). The highest rates of CRC are found in developed countries, such as the United States, Canada, Australia, New Zealand, and parts of Europe, while the lowest rates have been observed in particular Asian countries as well as in certain parts of

Africa and South America (Boyle and Langman, 2000, Jemal et al., 2011). In the United Kingdom, CRC is the third most frequent cancer after breast and lung cancers, with more than 36,000 cases diagnosed every year, and death rate is nearly 50% of that (Weitz et al., 2005). The prevalence of this cancer in both sexes is almost equal, though in recent statistics, it might be slightly increased amongst men (Jemal et al., 2011). The incidence of CRC escalates with age in individuals after the age of 40 years; further rapid increases have been found in individuals older than 50 years (Haggar and Boushey, 2009). Overall, the median age of patients with CRC at diagnosis is 71 years (Stec et al., 2011, Kohne et al., 2008).

1.2.8.3 Molecular Classification

The main advantage of tumour classification is to aid in the understanding of the pathogenesis and the biological behaviours of a specific tumour (Redston, 2001). Overall, classification of CRC is based on molecular characteristics such as microsatellite instability (MSI), chromosomal instability (CIN), and the CpG island methylator phenotype (CIMP; Figure 1-7) (Peltomaki, 2003). However, single molecular events are valuable identifiers for anticipating responses to targeted therapies that are contrary to those molecules (Ogino and Goel, 2008). An overlap between the three distinct molecular pathways in CRC pathogenesis has been reported. Thus, in a variety of cancers, there is more than one pathway. Yet, the importance of such an overlap has not been fully clarified (Sinicrope et al., 2006).

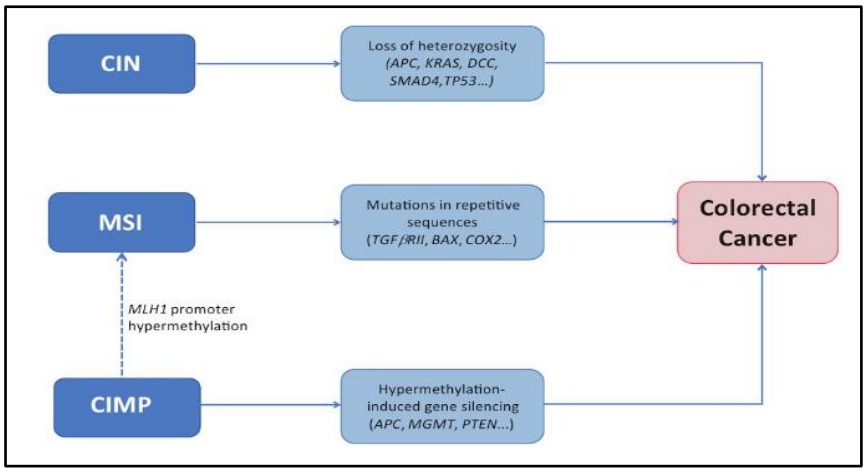


Figure 1-7. The mechanisms of genomic instability in CRC (Dheer, 2015)**.** CIN and CpG are the two main mechanisms of genomic instability. Nevertheless, a subset of roughly 10-15% of sporadic CRCs illustrates methylation of the mutL homolog 1 (MLH1) gene, leading to acquisition of a MSI phenotype.

1.2.8.4 Genetic and Epigenetic Modifications in CRC

1.2.8.4.1 Chromosomal Instability (CIN)

Recently, it was put forth that classes of genomic instability comprise subtle sequence alterations (e.g., changes, insertions or deletions and MSI), changes in chromosome number (i.e., aneuploidy), chromosome rearrangements and gene amplification (Grady and Carethers, 2008). CIN is the most common distinct phenotype in colon cancers, and it is present in 50-80% of CRCs (Goel et al., 2007). The determination of special patterns of chromosome gains and losses that take place throughout the colon adenoma–carcinoma sequence, as well as the notion that CIN is a primary event in cancer growth that escalates with cancer progression is consistent with the idea that CIN is a pathogenetic occurrence in CRC (Figure 1-8).

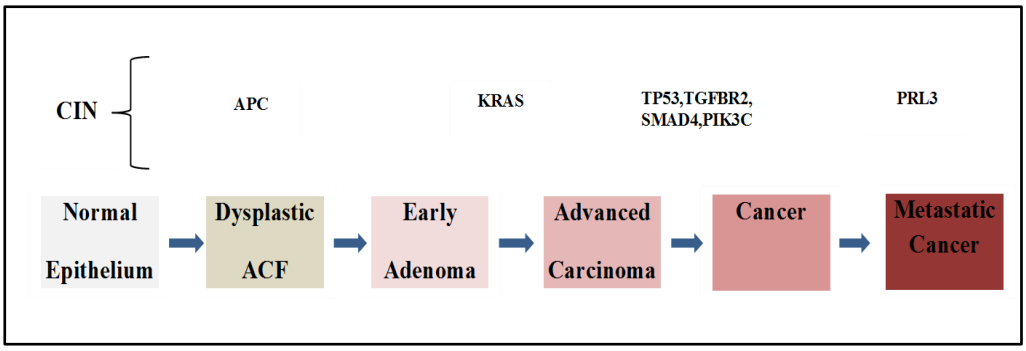


Figure 1-8. The main developmental stages of CRC with CIN (Grady and Carethers, 2008). The main quality of the CIN pathway is aneuploidy. Tumours start to occur with the interruption of the *Wnt* signalling pathway components, enclosing the presence of a somatic mutation in one allele and loss of heterozygosity of the second normal allele of the gene, *APC*, which is seen in dysplastic aberrant crypt foci (ACF). Development is then motivated through sequential waves of cellular clonal progress that obtain induced progress properties and contain mutational activation of the *KRAS* and *TP53* mutation with successive loss of heterozygosity of the remaining normal *TP53* allele, leading to the formation of carcinoma. In certain CRCs, the *PIK3CA* mutational activation takes place late in the adenoma–carcinoma sequence. Many TGF-signalling molecules are affected in the development of CIN, containing mutations in the kinase domain of transforming growth factor, beta receptor II (TGFBR2) and loss of heterozygosity (LOH) at chromosome 18q, specifically in the *SMAD4* and *SMAD2* locations. It is thought that many of the genes that are involved in metastasis comprise gene amplification of Protein tyrosine phosphatase 3 (*PRL3)*.

There are two ways of studying and evaluating CIN - DNA ploidy analysis and analysis of microsatellite markers for the LOH. The markers in the 18q region are generally more sensitive in LOH analyses than markers in other chromosomal regions, such as 1p, 2p, 3p, 5q, 8p and 17p (Rowan et al., 2005, Sugai et al., 2006). Nonetheless, CIN markers and features have not been standardized yet. LOH analyses are more likely to have either false-positive results because of polymerase chain reaction (PCR) bias and allele drop-out or false negative results arising from PCR bias or exposure of non-neoplastic cells to contamination. Uninformative results could appear as well in LOH analyses based on homozygosity in a specific marker or because of the absence of normal germ line DNA (Ogino and Goel, 2008).

Currently, array-based comparative genomic hybridization (array-CGH), as well as single nucleotide polymorphism (SNP) arrays, are being utilised to evaluate LOH and copy number gains/losses (Manning et al., 2010). In comparison with traditional CGH, these techniques are typified by high resolution in the assessment of DNA copy number gains and losses. However, the cost of the assay and the necessity of high-quality DNA are disadvantages. CRCs might have several common translocations with slight variations in allele copy numbers or DNA structure (Nowak et al., 2002). In addition, CRCs can be misclassified as CIN negative by copy number with different assays comprising array-CGH or LOH. The presence of several mechanisms of CIN - such as entire chromosomal LOH, mitotic recombination and mitotic gene alteration - is also evaluated thoroughly using array-CGH, SNP arrays and multi-colour fluorescence *in situ* hybridization (FISH) (Rowan et al., 2005). It is well-known that, as mentioned earlier, genetic alterations commence in an initial adenoma and accumulate to be eventually transformed to carcinoma. The CIN pathway involves activation of *KRAS* and inactivation of at least three tumour suppressor genes (TSGs), specifically loss of *APC*, *TP53* and LOH of chromosome 18q (Armaghany et al., 2012). These are discussed below.

1.2.8.4.2 APC

The *APC* gene is a tumour suppressor gene (Goss and Groden, 2000) that is localised to chromosome 5q21 by disease association in patients with FAP. It encodes a 312 kDa protein and 8.5 kb mRNA (Smith et al., 2002). Not only does the *APC* gene play a fundamental role in CRC pathogenesis in patients with FAP; it is also involved in most patients with sporadic CRC (Fearon and Vogelstein, 1990). Moreover, up to 80% of sporadic carcinomas of the large

intestines have been reportedly characterized by at least one mutation in the *APC* gene; a parallel frequency was found in colorectal adenomas. Therefore, a mutation in the *APC* gene is expected to be an initial event in the development of CRC (Bortlik et al., 2006).

The *APC* gene is a member of the *Wnt* signalling pathway, which is involved in progression and carcinogenesis (Kuraguchi et al., 2006). Functionally, it acts as a regulator for β-catenin levels in human tumours (Moon et al., 2004). The loss of *APC* is linked with stabilisation of the cytosolic β-catenin that eventually leads to its migration into the nucleus, consequently activating a cascade of events causing tumourigenesis. *APC* interacts with various cellular proteins, including axin-2, Asef or Rho guanine nucleotide exchange factor 4 (ARHGEF4), plakoglobin (JUP), the kinesin superfamily–associated protein 3 (KIFAP3), EB1 (MAPRE1) and the human homolog of drosophila discs large (DLG1). These interactions have led to the proposition that the *APC* gene regulates a number of cellular functions, such as intercellular adhesion, regulation of the cell cycle and apoptosis (Nathke, 2004, Fodde, 2003).

1.2.8.4.3 KRAS

The *KRAS* oncogene is a member of the Ras family proteins, featured prominently in transduction of mitogenic signals and signalling pathways of a number of functionally diverse molecules (Ilyas et al., 1999). This gene is located on chromosome 12p and encodes a 21-kD protein (p21ras) that functions in G-protein mediated signal transduction. *KRAS* is defined by its constitutive GTPase activity, which is lost once the gene has a mutation, most frequently at codons 12 and 13 (Smith et al., 2002). Mutations in *KRAS* result in an increase of cellular proliferation and malignant transformation. Approximately 50% of

sporadic CRCs have been found to be correlated with *KRAS* mutations (Kim et al., 2008). Consistent with the Fearon and Vogelstein colon cancer model, the *APC* gene is involved in adenoma establishment, while *KRAS* is part of the transition process from intermediate adenomas to carcinomas in sporadic CRCs. Thus, a somatic *KRAS* mutation is an initial event in colorectal carcinogenesis, mainly taking place throughout the transformation of a small to intermediate sized adenoma (Al-Kuraya, 2009, Ahmed et al., 2013).

Lee and colleagues (2008) evaluated MSI, CIMP and mutations of *KRAS* codons 12 and 13 and of *BRAF* codon 600 in more than 100 sporadic CRCs. They found a strong correlation between patients' survival and other clinicopathological variables, along with CIMP or genetic alterations. In addition, they reported that mutations in *KRAS* and *BRAF* were involved in 33.6% and 4.5%, respectively, of overall CRCs. Interestingly, the study findings uncovered a strong link with high CIMP, MSI and *BRAF* mutations, but not with *KRAS* mutations. Additionally, a worse clinical outcome was found for MSS-CRC with *KRAS/BRAF* mutation but not for those lacking a *KRAS/BRAF* mutation. These results indicate that the alleged poor clinical outcome of CIMP-high CRC patients is connected to the existence of the *KRAS/BRAF* mutation (Lee et al., 2008).

1.2.8.4.4 Phosphatidylinositol 3-Kinases (PI3KCA)

PI3KCA signalling pathways are significant in carcinogenesis, comprising those in apoptosis, migration, and proliferation (Wymann and Pirola, 1998). *PIK3CA* is an important member of the lipid kinase family, encoding the p110α catalytic subunit of phosphatidylinositol 3-kinase (Wang et al., 2007a). Two key mutational hotspots are found in exons 9 and 20 of the gene, which enclose

crucial nucleotides of the helical and catalytic domains, respectively (Vanhaesebroeck and Alessi, 2000b). Activating point mutations in these regions elevate kinase activity, activate *AKT* and are associated with carcinogenesis via reduced apoptosis, loss of contact inhibition and raised tumour invasion (Wang et al., 2007a). *PIK3CA* has been observed to be mutated in up to 30% of CRC cases (Armaghany et al., 2012), and this is correlated with poorer patient outcomes. The *PIK3CA* mutation frequently co-occurs with the *KRAS* mutation, which enhances *AKT* pathway signalling as well as cellular transformation more than the *PIK3CA* mutation alone (Wang et al., 2007a). Activating mutations in *PIK3CA* is considered a novel mechanism for inducing *PI3K* signalling (Samuels et al., 2004). Amongst patients with wild-type *KRAS* CRCs, *PIK3CA* mutations have recently related to a substantial increase in mortality because of colon cancer. On the other hand, the activation of the PI3K/AKT pathway can be significant in tumour aggressiveness in a background where the *KRAS* is not constitutively activated by a somatic mutation. (Ogino et al., 2009b). Lastly, phosphatase and tensin homolog (PTEN) is a phosphatase that regulates the *PI3K/AKT* signalling pathway by dephosphorylating phosphatidylinositol3,4,5 trisphosphate (PIP3) to inhibit activation of *AKT* through hyperactivation of *PI3K* signalling (Yin and Shen, 2008).

1.2.8.4.5 18q LOH

Allelic loss at chromosome 18q is considered a late event in the colorectal carcinogenic process, and it can be evaluated by LOH analysis (Ogino et al., 2009a). LOH has been reported in a high proportion of CRCs, and the 18q deletion is associated with more aggressive clinical behaviour of CRCs

(Armaghany et al., 2012). There are a few TSGs in 18q, including deleted in colorectal carcinoma (DCC), *SMAD4* (DPC4), *SMAD2* and Cdk5 and Abl Enzyme Substrate 1 (*CABLES1*) (Ogino et al., 2009a). 18q LOH has been reported to be inversely related to MSI (Watanabe et al., 2001), which, just as stated before, is a significant molecular classifier in CRC (Jass, 2007). Several studies have described that the occurrence of tumoural 18q LOH is associated with poorer survival in patients with CRC (Watanabe et al., 2001, Jen et al., 1994). Further, other studies that have assessed the prognostic significance of 18q LOH (Ogunbiyi et al., 1998, Carethers et al., 1998, Jernvall et al., 1999) did not take into account the likely confounding effect of MSI, which is normally associated with increased patient survival (Popat et al., 2005).

1.2.8.4.6 TP53

TP53 is a tumour suppressor gene located on the short arm of chromosome 17 and is one of the most frequently mutated genes in a wide range of cancers (Goh et al., 1995). It encodes a nuclear phosphoprotein with the ability to bind to DNA and act as a transcriptional activator. Genes that are activated by *TP53* affect cell cycle arrest, and as a consequence, damaged cells prioritise repairing themselves lest apoptosis takes place (Steele et al., 1998). Mutations of *TP53* have been found to be associated with nearly 50% of CRC (Goh et al., 1995), particularly with lymphatic dissemination (Goh et al., 1994) and possibly with worse patient prognosis (Hamelin et al., 1994). Most point mutations identified in *TP53* genes represent missense mutations, which lead to amino acid substitutions in the *TP53* protein (Goh et al., 1995). Mutations of the *TP53* gene can be identified by immunohistochemical analysis at the protein level or by sequencing at the gene level; the latter technique is confirmed to be more accurate, though is more

complicated (Bunz et al., 1999). Furthermore, *TP53* mutations predict reduced sensitivity of cancer cells to most chemotherapeutic agents, including fluorouracil. However, more recent findings contradict the issue of its prognostic and predictive roles in CRC (Hoff, 2005).

1.2.8.4.7 Microsatellite Instability (MSI)

Microsatellite instability is a common molecular occurrence and represents the other major pathway in genomic instability in CRCs. It is caused by the loss of DNA-MMR activity (Vilar and Gruber, 2010). It is categorised by the existence of length variation in nucleotide repeat sequences recognized as microsatellite sequences (Peltomaki, 2003). MSI has been detected in different tumours, including gastric, CRC, ovarian and sebaceous carcinomas in addition to glioblastoma and lymphomas (Boland et al., 1998, Pal et al., 2008). Furthermore, the MSI phenotype serves as the hallmark of defective MMR as it was discovered in CRC, specifically when it was demonstrated that germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* are responsible for a genetic predisposition to CRC, known as HNPCC or Lynch syndrome (Vilar and Gruber, 2010). Approximately 15% of sporadic (i.e. non Lynch Syndrome) CRCs have instability in at least two of the standard microsatellite markers. About 3% of these have been linked with Lynch syndrome, while the remaining 12% with sporadic, acquired hypermethylation of the *MLH1* promoter, which takes place in tumours with the CIMP (Walther et al., 2009). CRCs with MSI have specific traits (e.g., the propensity to be present in the proximal colon), show lymphocytic infiltration and be poorly differentiated (Boland and Goel, 2010). The prognosis of CRC with MSI is better than colorectal MSS, but that

does not mean MSI cancers have the same response to chemotherapeutic schemes utilized for treatment of MSS cancers (Ng and Schrag, 2010).

MSI tumours are divided into two sets according to the proportion of instability in certain proposed loci-microsatellite instability-high (MSI-H) and microsatellite instability-low (MSI-L) (Ward et al., 2001). MSI-H tumours are noted when instability is seen in two or more markers out of five tested loci, while MSI-L tumours are defined when instability occurs in just one marker (Lindor et al., 2002). Furthermore, in cases where more than five loci are tested, MSI-H is identified when the instability of loci is ≥ 30%, while a lesser percentage (< 30%) of loci instability is identified as MSI-L (Boland et al., 1998). The role of MSI-L in cancer is still vague and incompletely understood (Walther et al., 2009). Microsatellites are known for repetitive sequences that are disseminated through the human genome and contain mononucleotide, dinucleotide or higher-order nucleotide repeats, such as $(A)_n$ or $(CA)_n$. These sequence motifs are specifically disposed to mutation accumulation as DNA polymerases cannot bind DNA properly during the process of DNA synthesis (Ellegren, 2004). The most common errors related to microsatellites are base–base and insertion–deletion loops. These unpaired nucleotides come about once the first nucleotide and template strand detach and incorrectly reanneal in a microsatellite. Studies have confirmed that insertions or deletions in microsatellites found in DNA coding regions are able to create frameshift mutations, which could result in protein truncations (Jiricny, 2006).

The MMR system is essential for error correction in microsatellites (Figure 1-9) (Vilar and Gruber, 2010). The most important proteins involved in this system work as heterodimers and include MLH1, MutS protein homolog 2 (MsH2), MutS Homolog 3 (MsH3), MutS homolog 6 (MsH6) and postmeiotic segregation increased 2 (PMs2) (Vilar and Gruber, 2010). Once a mismatch is identified, several steps ensue - MsH2 interacts with MsH6 to form a Mutsα complex or with MsH3 to form a Mutsβ complex, and MLH1 links with PMs2, PMs1 or MLH3 to develop MutLα, MutLβ or MutLγ complexes, respectively (Boland and Goel, 2010, Vilar and Gruber, 2010). To recognise mismatches and insertion–deletion loops, a complex comprising Muts and a MutL acts in tandem with replication factor C. Proliferating cell nuclear antigen (PCNA) and exonucleases 1 (EHO1) perform the function of excising the mismatch from the DNA sequence. As a final point, DNA strands can be resynthesized and religated by DNA polymerase δ and DNA ligase (Hoeijmakers, 2001, Jiricny, 2006). When genes responsible for the recognition step are mutated, there is an accumulation of errors in DNA, leading to MSI (Vilar and Gruber, 2010).

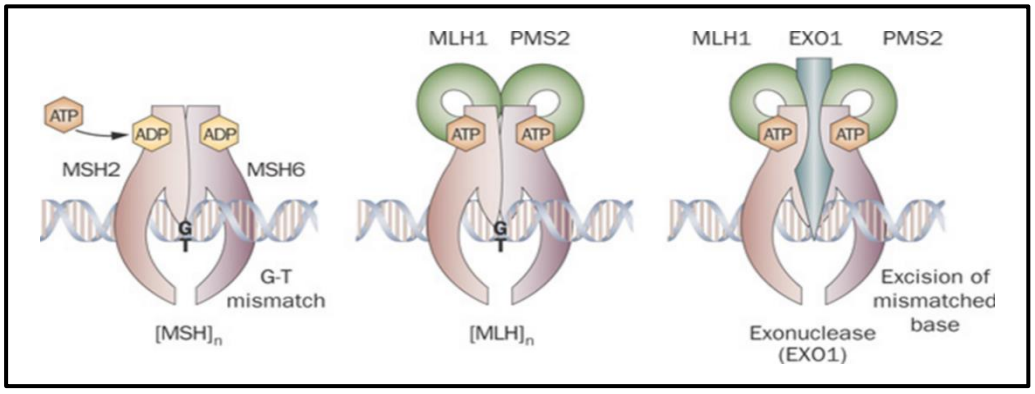


Figure 1-9. The mechanism of mismatch repair (Vilar and Gruber, 2010). As soon as a mismatch is identified, MSH2 interacts with MSH6 or MSH3, and MLH1 links with PMS2, PMS1 or MLH3. Mismatches and insertion–deletion loops are recognised by the presence of a MutS and a MutL complex. Elimination of the mismatch is accomplished by EXO1 and/or PCNA.

1.2.8.4.8 CpG Island Methylator Phenotype (CIMP)

More than 50% of human genes' promoters are embedded in CpG islands and are prone to methylation-associated silencing. However, there are genes that are more regularly affected than others (McCabe et al., 2009). CpG islands normally include 500–2000 bp of the promoter regions but can also be prolonged to 5'-untranslated regions within the first exon of various TSGs (Boland and Goel, 2010). Furthermore, and again, just as described previously, one of the most prominent mechanisms behind CRC carcinogenesis is CIMP. It is believed that CIMP can be a cause of MSI-H sporadic CRCs, but its role in MSS tumours is debated (Goel et al., 2007). Roughly 35–40% of CRCs display CIMP (Goel et al., 2003). A few recently published studies have contended that CIMP is a vital mechanism for gene inactivation in carcinogenesis. Furthermore, there is the robust suggestion that several TSGs, such as *p14*, *p16*, *MGMT* and *hMLH1*, are silenced by promoter methylation in CRC (Rashid and Issa, 2004).

CIMP, as a subtype of CRCs, was initially discovered using a set of hypermethylated genomic loci that were found in tumours. It has been hypothesised to precede the "mutator phenotype" in the progression of MSI tumours. Interestingly, sporadic CRC without CIN and CIN-/MSI-phenotypes has been found associated with CIMP (Goel et al., 2007), indicating that CIMP and CIN represent two different mechanisms of genetic and epigenetic instability. CIMP is referred to as an epigenetic phenomenon, as this does not include a persistent alteration (e.g., a point mutation or deletion) in the sequence of target DNA. Instead, it merely reflects a likely reversible change caused by methylation of the cytosine nucleotides that take place in the framework of the CpG dinucleotide (Boland and Goel, 2010). Many studies have suggested that

the designation of a CIMP is an event with a normal distribution of methylation level and that it might rely on age or MSI. However, the observation of such a phenotype has been acknowledged using different methylation markers (Hawkins et al., 2002, Yamashita et al., 2003). Wiesenberger and colleagues (Weisenberger et al., 2006), for example, screened 195 CpG island methylation markers using a quantitative MethyLight method, and a new panel of vigorous markers was put forth to stratify CIMP tumours. These studies have shown that CIMP associates with DNA mutations and certain clinicopathologic features, such as MSI, mutations in *BRAF* and *KRAS* and appearance on the proximal colon. The molecular mechanism responsible for CIMP has not yet been established (Boland and Goel, 2010). Much of the research in this field supports the notion that there is a "methylator phenotype"; however, other studies dispute this (Yamashita et al., 2003).

1.2.8.5 Molecular Biomarkers in CRC

The rapidly growing understanding of the molecular biology, gene sequencing and molecular diagnostics of CRC have provided important information for the identification of molecular markers with the objective of developing tailored therapeutic regimens (Deschoolmeester et al., 2010). Nevertheless, no molecular marker has yet been introduced into clinical practice. With the likely exception of MSI, molecular markers have not even effectively cleared the stage of replication in independent patient groups, hindering any initiatives at prospective validation (Tejpar, 2007). Obviously, there is a necessity for robust and independent prognostic markers that can accurately discriminate patients into subclasses for which diverse treatment choices, composed of the likelihood of neoadjuvant treatment, are appropriate (McDermott et al., 2011).

Furthermore, markers to prospectively predict response or resistance to particular treatments as well as markers to detect patients that are likely to experience serious toxic side effects from these therapies are required (Duffy and Crown, 2008). Recently, much research has been dedicated to studying new biological prognostic/predictive markers in CRC (Table 1-1). A number of these markers are being investigated, but only a small number have adequate evidence to permit their use in clinical practice (Deschoolmeester et al., 2010). Moreover, different standards must be met to confirm that a biomarker is clinically valuable, including demonstration from diverse independent studies.

Table 1-1. The most studied prognostic/predictive markers in CRC (Deschoolmeester et al., 2010).

Oncogenes	*KRAS*, *EGFR*, c-*myc*, *TGF* and *BRAF*
TSGs/LOH	*TP53*, *p27*, *p21*, *MCC*, 18q LOH deleted in CRC (*DCC*), *SMAD2*, *SMAD4*, 1p LOH, 8p LOH, 9p LOH and 14p LOH
Proliferating indices	Proliferating cell nuclear antigen, Ki-67 and MIB-1
Genetic instability	MSI, CIN, CIMP and thymidylate synthase
Apoptosis	Bcl-2, BAX, antioxidants, Fas/CD95, PI3K, catalytic and α polypeptide
Angiogenesis	VEGF, microvessel density, TP, CAMs and TSP-1
Metastasis and invasion	MMPs, uPA, PAI-1, CD44, E-cadherin and NM23A

1.2.9 Pancreatic Cancer:

1.2.9.1 Epidemiology

Pancreatic cancer (PC) is one of the top five most frequent cancers causing death worldwide. It has been found responsible for nearly 4% of all cases of mortal cancer (Zhang et al., 2016). Despite enormous improvement in therapeutic efficiency, the mortality rate remains high versus diagnosed cases of PC (Chu et al., 2010). Globally, in 2015, an estimated 367,411 new cases were diagnosed with PC and 359,335 died from this disease. In 2011, in the United Kingdom, PC was the fifth most common cause of cancer death according to Cancer Research UK, and every day, 24 new cases were diagnosed, and almost the same number of people died (Zhang et al., 2016). These statistics mirror the advanced stage at which majority of patients with PC are diagnosed and the lack of effective chemotherapeutic treatments for advanced disease.

1.2.9.2 Molecular Epidemiology

The pathology and molecular properties of advanced stages of PC are generally indefinite. Multiple genetic syndromes, including hereditary pancreatitis (HP), HNPCC, Peutz-Jeghers syndrome (PJS), familial breast cancer and familial atypical multiple mole melanoma (FAMMM), have been found to be linked to an increased risk of PC. It was thought for a long time that working in industrial fields increased the likelihood of exposure to carcinogens and that this might be a critical element of pancreatic cancer aetiology, yet there is inadequate support for this idea as the primary contributors for PC continue to be under investigation. Researchers have attempted to quantify the risk factors of PC and

people at high risk in order to encourage the ultimate prevention of this disease (Li et al., 2004).

1.2.9.3 Molecular Pathology

The understanding of the pathogenesis of PCs has become significantly more sophisticated because of the improvements in molecular biology. Likewise, just as in the case of other threatening malignant diseases, pancreatic ductal carcinoma initiates from the build-up of accumulated mutations (Table 1-2). Based on histological data and molecular genetic profiles, a growth model has been developed that defines pancreatic ductal carcinogenesis - the pancreatic ductal epithelium advances from normal to expanded pancreatic intraepithelial neoplasia (PanIN), then to invasive tumours (Li et al., 2004) (Figure 1-10). Most PCs are sporadic, while between 5% to 10% of patients with PC have a family history. Since the first identification of mutation of the *KRAS* oncogene, incredible progress has been made in understanding the genetics of PC. Over 85% of pancreatic ductal tumours have been observed to possess an activating point mutation in the *KRAS* gene at the initial stage of pancreatic cancer growth. Further, detection of *KRAS* mutations in the duodenal juice, pancreatic juice and stool of patients with pancreatic malignancy has been proposed as an early detection policy (Wenger et al., 1999, Li et al., 2004).

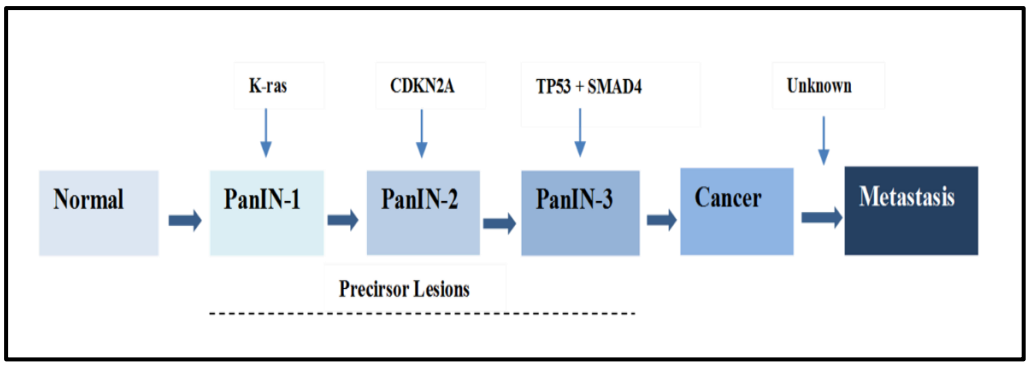


Figure 1-10. The genetic alterations model of pancreatic tumourigenesis (Yachida and Iacobuzio-Donahue, 2013). The molecular changes that take place throughout pancreatic tumourigenesis is categorised into primary (activating mutations of *KRAS* in PanIN-1), intermediate (inactivating mutations of *CDKN2A* in PanIN-2) and late (inactivating mutations of *TP53* and *SMAD4* in PanIN-3) events. Additional mutations in other genes could occur throughout PanIN development, but have not been depicted in this model.

The p16 TSG is inactivated in roughly 95% of pancreatic tumours, and this normally transpires during the advanced phase of pancreatic carcinogenesis (Schutte et al., 1997). The second most frequently inactivated TSG is *TP53*, which is mapped on chromosome 17p. Its inactivation process is also initiated during a late stage of tumourigenesis. The *MADH4* gene (*SMAD4* or *DPC4*) has also been observed to be inactivated in 55% of pancreatic adenocarcinomas. *MADH4* inactivation is a later occurrence in pancreatic tumourigenesis, similar to *TP53*. Additional genetic modifications have also been noted to be implicated in pancreatic cancer development, but they are less common. In a thorough mutational analyses of 42 pancreatic ductal tumours, Rozenblum et al., established that all the tumours contained mutations in *KRAS*. The distinct mutational rates of TSGs, *p16*, *TP53*, *MADH4* and *BRCA2*, were 82%, 76%, 53% and 10%, correspondingly (Rozenblum et al., 1997, Li et al., 2004).

Table 1-2. The frequently transformed oncogenes and TSGs in pancreatic adenocarcinoma (Li et al., 2004).

Marker	Chromosomal location	Modification rate (%)
	Oncogenes	
KRAS	12p	75–100
HER2/neu	17q	65-70
AKT2	19q	10-20
MYB	6q	10
	Tumour suppressor and genome maintenance genes	
TP53	17p	40–75
CDKN2A	9p	27–98
CDKN2A	9p	27–82
CDKN2B	9p	27–48
MADH4	18q	50–55
FHIT	3p	66–70
RBI	13q	0–10
BRCA2	13q	7–10
STK11	19q	5
MAP2K4	17p	4
ALK5	9q	1
TGFBR2	3p	1
TGFBR2	3p	3
MLH1	3p	3

1.2.10 Liver Cancer

1.2.10.1 An Overview

Liver cancer is ranked the fifth most common cancer in the world with respect to prevalence and mortality (Hagymasi and Tulassay, 2008, Ozen et al., 2013). Hepatocellular carcinoma (HCC) represents 85-90% of liver cancers, and approximately 70-90% of HCC occurrence is highly associated with an established background of chronic liver disease and cirrhosis. Most HCCs are associated with hepatitis B and C, as well as aflatoxin exposure (McGlynn and London, 2011). Like other types of cancer, liver cancer development is through a chain of processes (Hagymasi and Tulassay, 2008, Ozen et al., 2013).

1.2.10.2 Molecular Abnormalities in Liver Cancer

Earlier studies (Ozen et al., 2013, Hagymasi and Tulassay, 2008, Liu et al., 2014) examining the entire genome for HCC demonstrated a high number of mutated genes as well as common genes, like *TP53*, *CTNNB1*, *AXIN1* and *CDKN2A*. Aside from *CTNNB1* changes, a large portion of other mutations apparently lead to loss of function in genes. Therefore, HCC-related mutations cannot be effectively targeted for treatment. With this, epigenetic changes that are frequent might serve as new targets. The well-known epigenetic alterations in HCC pathogenesis encompass DNA hypomethylation, promoter methylation, aberrant expression of non-coding RNAs and dysregulated expression of additional epigenetic regulatory genes such as *EZH2*. The multiple cancer hallmarks and the primary molecular abnormalities in the development of HCC are summarized in Figure 1-11.

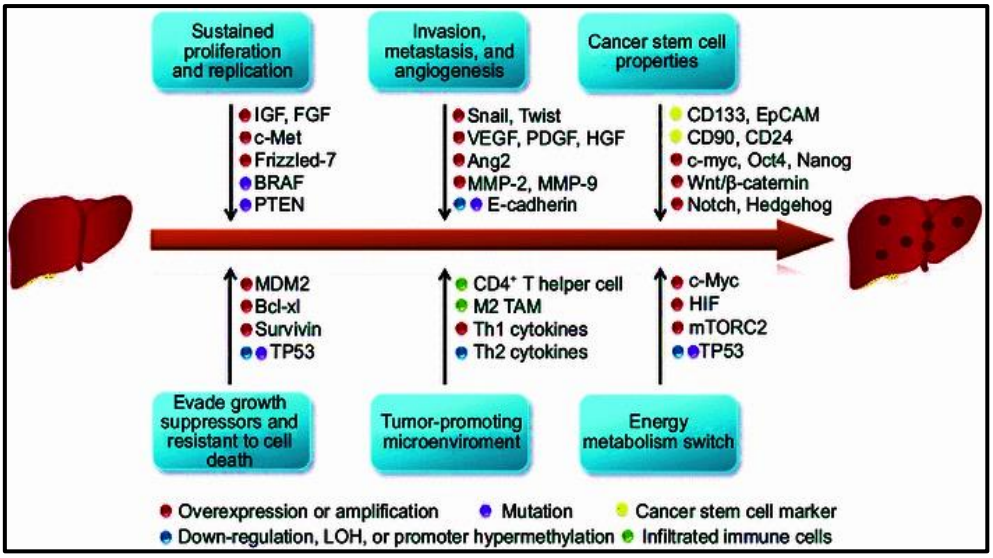


Figure 1-11. Main molecular abnormalities in the development of HCC (Liu et al., 2014). HCC development follows a multi-step process that comprises several cancer hallmarks along with cellular and molecular changes.

1.2.11 Lung cancer

1.2.11.1 Epidemiology and Risk Factors

Currently, lung cancer has become one of the major life-threatening diseases across the world, and especially for men (Rafiemanesh et al., 2016, Dela Cruz et al., 2011, Yoder, 2006a). Based on recent statistics, the death rates from lung cancer are believed to reach up to 3 million per annum by 2035 globally (Bilello et al., 2002). Several ecological and clinical studies have confirmed that there is a robust connection between tobacco smoking and lung cancer incidence. This means that an elevation in tobacco consumption leads to increased lung cancer mortality, particularly in developing countries (Didkowska et al., 2016). Further crucial risk factors also play a role in the development of lung cancer, and these consist of environmental exposure to chemicals, like radon, industrial cancer-causing agents, and pre-existing non-malignant lung diseases. In addition, molecular biology studies have elucidated the role of genetic factors in

predisposing an individual for lung cancer. Despite the immense efforts put towards developing chemopreventive agents, avoidance of smoking and promotion of smoking cessation are the best policies to diminish lung cancer incidence. Unlike other cancers, lung cancer seems to have a solid epidemiological connection between a preventable behaviour and the rate of disease incidence (Bilello et al., 2002).

1.2.11.2 Types of Lung Cancer

Lung cancer has been classified into two main categories - small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter is further demarcated into squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Based on the fact that therapeutic efficacy varies significantly depending on the type and progression stage of lung cancer, accurate diagnosis is vital for determining the precise type of cancer, its stage and the ability of the affected individual to endure therapy. NSCLC accounts 80% of all lung cancers; adenocarcinoma accounts for 40% of all cases of NSCLC. The most frequent event in the central sector of the lung is squamous cell carcinoma, while adenocarcinoma tumours are peripheral in regards to where they start, originating from either the alveolar surface epithelium or bronchial mucosal glands. Large cell carcinoma represents just 15% of all lung cancers and appears to be decreasing in incidence through appropriate early diagnosis. SCLC is the second most significant type of lung cancer, wherein there are additionally numerous histologic groupings - pure small cell, mixed small cell and large cell carcinoma, along with combined small cell. Typically, SCLC is more aggressive than NSCLC and is observed as a central lesion with hilar and mediastinal invasion together with regional adenopathy. The probability of distant

metastasis at presentation is high among patients with SCLC, and the most widely documented destinations of metastasis of lung cancer are the bones, liver, adrenal glands, pericardium, cerebrum and spinal cord (Yoder, 2006b).

1.2.11.3 Molecular Changes during Lung Cancer Pathogenesis

The carcinogens present in tobacco or other ecological poisons prompt the loss of the 3p21.3 allele in a huge number of cells at various points of the respiratory epithelium. Later, TSGs situated on the 3p21.3 chromosome arm develop haplo-insufficiency (a phenotype whereby a diploid organism has lost one copy of a gene and is left with a single functional copy of that gene). The subsequent hit occurs in genes that are required for cell proliferation, like, for example, *TP53*, p16 or other genes either by mutational inactivation or by promoter hypermethylation. This allows for clonal growth of the primarily transformed cells. It has been demonstrated that molecular pathogenesis varies significantly between SCLC and NSCLC (Zabarovsky et al., 2002). It is proposed that amidst pathogenesis of SCLC, neoplastic cells emerge directly either from typical or hyperplastic epithelial cells without going through characteristic preneoplastic intermediate pathological stages (parallel theory of lung cancer pathogenesis). In contrast, NSCLC pathogenesis is guided by sequential morphological changes (sequential theory). The model of lung tumour pathogenesis is summarised in (Figure 1-12) (Rom et al., 2000, Hirsch et al., 2001).

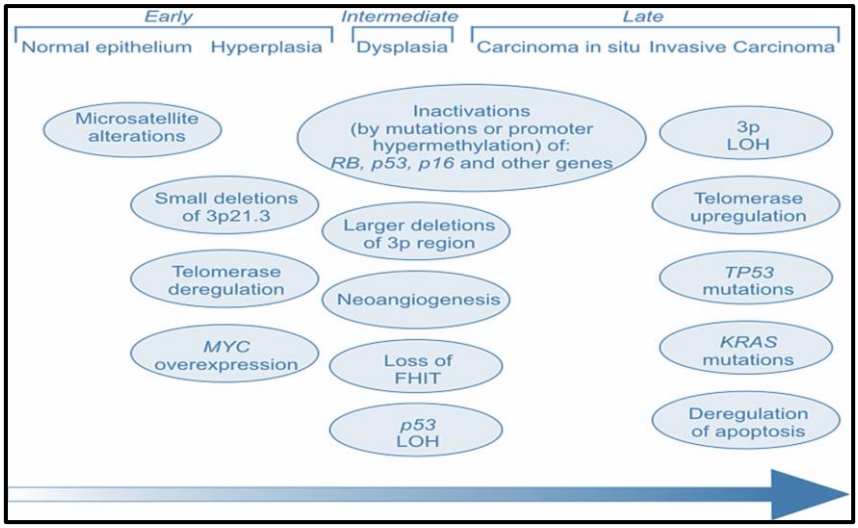


Figure 1-12. Molecular changes during lung cancer pathogenesis.

1.3 The Aims of the Study

There is a growing body of literature that identifies the importance of CD24 in cancer tumourigenesis, invasiveness, metastasis, and apoptosis; however, the underlying signalling mechanisms remain unclear. Therefore, the main goal of the current study was to determine:

1. The mechanism by which CD24 affects cell function
2. The mechanisms by which CD24 expression could be controlled in cancer cells.

This includes:

- Determining the possible downstream target molecules of CD24.
- Establishing the possible upstream regulator molecules of CD24.
- Identifying the potential signalling pathways associated with CD24.
- Ascertaining the role of CD24 in EMT signalling.

Four cancer model systems (colorectal, pancreatic, liver and lung cancer) were employed to achieve these objectives.

2 MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell Lines

The experimental work was performed in four cancer models: colorectal cancer, pancreatic cancer, liver cancer, and non-small cell lung cancer. For colorectal cancer, the six cell lines used, HCT116, RKO, SW620, HT29, DLD-1 and GP2D, were obtained from the Molecular and Population Genetics Laboratory, London Research Institute, Cancer Research, London, UK. For pancreatic cancer, the three cell lines used, COLO357, PSN-1 and Panc-1, were gifted to our group by Professor William R. Otto, Histopathology Unit, London Research Institute, Cancer Research, London, UK. For non-small cell lung cancer, the three cell lines used, A549, H460 and H226, were given to our group by Professor Sue A. Watson, Division of Pre-Clinical Oncology, Queen's Medical Centre, University of Nottingham, Nottingham, UK. For liver cancer, a cell line used, HUH7, was kindly gifted to us by Dr Alex Tarr, Division of Virology, Queen's Medical Centre, University of Nottingham, Nottingham, UK. Cancer cell lines and their characteristics are listed in Table 2-1. All cell lines used were underwent regular checks for mycoplasma infection. The mutation status of the cell lines for frequently mutated genes in cancer was validated using HRM (Appendices: Tables 9-1 & 9-2 and Figures from 9-5 to 9-16).

2.1.2 Cell Maintenance

CRC cell lines, NSCLC cell lines and pancreatic cell lines, PSN-1 and COLO357, were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco®, Invitrogen, UK). The pancreatic cancer cell line, Panc-1, and liver cancer cell line, HUH7, were grown in Roswell Park Memorial Institute (RPMI-

1640; Sigma, USA) media. Both culture media were supplemented with 10% foetal bovine serum (FBS) (Sigma, UK), with 100 unit/ml penicillin and 100 μg/ml streptomycin (Invitrogen, UK). The cells were maintained at 37°C in a 5% CO_2 atmosphere. All cell culture procedures were carried out in a class II laminar flow cabinet under sterile conditions. The cells were regularly examined under the microscope to assess their viability and ensure that there were no potential fungal or bacterial infections within the cultural media. Cells were nourished twice weekly by replacing the culture media with fresh media.

2.1.3 Cell Thawing and Growing

Once retrieved from liquid nitrogen (LN_2) and defrosted in a water bath, cells were mixed gently and transferred into 20 ml sterilised universal tubes (Thermo Scientific, UK) containing 7 ml of growing medium (DMEM or RPMI-1640 supplemented with 10% FBS and 100 unit/ml penicillin and 100 μg/ml streptomycin). Cells were centrifuged for 5 min at 1500 rpm, re-suspended with 7 ml of medium and cultured in 25 cm^2 (T25) culture flasks (Costar, UK) labelled with the cell type, passage number, date, and initials. The cells then incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2.

2.1.4 Cell Passage (Splitting)

Cells were typically allowed to reach up to 90-100% confluence before being washed twice with 5 ml of phosphate buffered saline (PBS) (Sigma) and incubated for 5 min with 2 ml of trypsin-EDTA (1X) to detach them from the surface of the tissue culture flask. The cells were then re-suspended in 8 ml of full fresh DMEM or RPMI-1640 to inhibit the trypsin activity. Next, the cells were transferred into a sterile tube and centrifuged at 1500 rpm for 5 min, the

supernatant was discarded and the pellet was re-suspended in 10ml of DMEM or RPMI-1640. The cell suspension was then placed into a new flask 75 cm^2 (T75) containing the appropriate volume of full media at a 1:10 split ratio. Cell lines were used for experimental work up to a maximum of 10 passages after retrieval from the freezer.

2.1.5 Cell Counting

To calculate the number of cells required for the experimental work, the cells were counted using the dye-exclusion method whereby viable cells (with an intact cell membrane) do not take up a dye in the medium. Firstly, the cell suspension was re-suspended gently and then 50 µl of the mixture was added to an equivalent amount of Trypan blue (TB) dye (Sigma) in an Eppendorf tube (1.5 ml), mixed well and incubated for 5 min at room temperature. Twenty µl of the mixture was pipetted into the Neubauer chamber (haemocytometer) for counting of the live cells (which did not take up TB dye) using a microscope (Zeiss, Germany) at 10× magnification. The cells were counted in 4 sets (of 16 corners) and were multiplied by 10^4 (the size of 1 large corner squire) and then multiplied again by 2 (the dilution factor) to give the total cell count per ml. Later, an automated cell counter (BIO-RAD, TC20) was also used alongside the ordinary method for cell counting.

2.1.6 Cell Freezing

The cells were grown to confluence (100%), the media aspirated and cells washed twice with PBS, trypsinized and centrifuged as described earlier. A mixture containing 3.6 ml of 90% FBS, 400 µl of 10% dimethyl sulphoxide (DMSO) for freezing the cells was prepared in a sterile universal tube and mixed

thoroughly. The cell supernatant was aspirated and the pellet re-suspended in the "freezing" mixture. Cryo-Vials (Simport Scientific, Canada) were labelled with the cell name, passage number, date, and initials, and 1 ml of the cell suspension was transferred into each corresponding cryo-vial. Cryo-vials were then transferred to the Nalgene® Cryo 1°C "Mr. Frosty" Freezing Container (Thermo Scientific) and placed into the freezer at -80°C. After 24 to 48 hrs, the cryo-vials were taken out of the freezing container and placed into a labelled box at -80°C, with some stored in LN_2 at -196°C.

2.1.7 Cell Lysis and Protein Extraction

Briefly, culture media was aspirated from the 6-well plate (Corning Incorporation, Coaster, UK) and cells were then washed twice with an appropriate volume of PBS (1X), treated with an appropriate volume of ice-cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) containing a Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) (Thermo Scientific) at a 1:100 dilution. The plate was incubated on ice for 15 min and the resulting protein lysates were transferred into 1.5 ml Eppendorf tubes and centrifuged at 13,000 rpm for 30 min at 4°C. Finally, the protein lysate was transferred in a new Eppendorf tube, aliquoted, and stored at -20°C for further quantification.

2.2 Protein Quantification

The Pierce® bicinchoninic acid (BCA) Protein Assay kit (Thermo Scientific, UK) was used for protein quantification. A series (25 to 2000 µg/ml) of bovine serum albumin (BSA) standard solutions (Sigma) were prepared using RIPA buffer as a diluent according to the manufacturer's instructions. Next, 12.5 µl of

BSA standards and the proteins were added into a 96-well plate (Corning Incorporation) in duplicate, followed by 100 µl of the working reagent (a ratio of 1:50 reagent A: reagent B). The plate was shaken for 10 sec using a LabSystems Multiskan EX microplate reader (Thermo Scientific) and incubated at 37°C for 30 min. The absorbance (i.e. optical density) for each well was read at 550 nm using a LabSystems Multiskan EX microplate reader. The standard curve for optical densities of BSA standards was plotted using IBM SPSS Statistics v22.0 software (US) for the determination of the protein concentration.

Table 2-1. The characteristics of the cancer cell lines used

(A) Colorectal cancer cell lines						
Cell Line	**Age**	**Sex**	**Dukes' Stage**	**Differentiation**	**Site**	**Growth Mode**
HCT116	64	M	D	Well	Colon	Adherent
RKO	64	F	C	Poor	Colon	Adherent
SW620	51	M	C	Moderate	Colon, lymph node	Adherent
HT29	44	F	B	Moderate	Colon	Adherent
DLD-1	Adult	M	C	Moderate	Colon	Adherent
GP2D	71	F	B	Poor	Colon	Adherent
(B) Pancreatic cancer cell lines						
Panc-1	56	M	n/a	Poor	Pancreatic duct	Adherent
PSN-1	n/a	M	n/a	-	Pancreas	Adherent
COLO357	n/a	n/a	n/a	Well	Lymph node metastasis	Adherent
(C) Lung cancer cell lines						
H460	n/a	M	n/a	Well	Lung, pleural effusion	Adherent
H226	n/a	M	n/a	Well	Lung, pleural effusion	Adherent
A549	58	M	n/a	Poor	Lung	Adherent
(D) Liver cell lines						
HUH7	57	M	n/a	well	Liver	Adherent

2.3 Mutation Screening for the Cell Lines

2.3.1 Quick-Multiplex-Consensus (QMC)-PCR

2.3.1.1 Primer Design

The identity of the cell lines was validated by screening for mutations known to be present in the cell lines. Screening was performed using the in-house QMC-PCR assay followed by High Resolution Melting (HRM) analysis. QMC-PCR is a nested process comprising a primary pre-diagnostic multiplex (PDM) reaction followed by a singleplex specific diagnostic (SSD) reaction (Fadhil et al., 2010). The PDM reaction included outer primer pairs for several variant target classes in a single reaction. The SSD reaction only contained a single inner primer pair precise to each target. Suitable primers were designed using Primer3 software (http://www.bioinformatics.nl/cgi-bin/ primer3plus/primer3plus.cgi); for the PDM reaction, primers were verified in silico PCR (http://genome. csdb.cn/cgi-bin/hgPcr/) and non-cross-reacting primers were selected. Primers covered hotspots in *KRAS* (exons 3 and 4 including codons 61 and 146, respectively), *BRAF* (exons 11 & 15 (E11 including codon 600), PI3KCA (exons 9 and 20), PTEN (exons 3 & 8), SMAD4 (exons 9 & 11), and PT53 (exons 2 & 6). Primers were designed to achieve a maximum PCR product size of approximately 200 bp.

2.3.1.2 Thermal Cycling Conditions

A thermal cycling system was used for all the PCRs conducted. The PDM reaction was carried out in a final volume of 25 µl, with each reaction comprising 1X HotShot master mix, 10 primer pairs (for every hotspot) at a final

concentration of 0.4 µM for each primer and 20 ng template DNA. PCR was performed using a dual-step protocol: 1 cycle of 95^0C for 5 min, followed by 25 cycles of 95^0C for 1 sec and 55^0C for 1 sec. The incubation time took 38 min. The SSD reaction was performed in a final volume of 10 µl, containing 1X HotShot master mix, 1 primer pair with each primer at a final concentration of 0.25 µM and 1X LC Green PLUS. The template contained 1µl of a diluted product (1:100) from the PDM reaction and the PCR was carried out using a twostep protocol: 1 cycle of 95^0C for 5 min, followed by 45 cycles of 95^0C for 1 s and 55^0C for 1 s. The duration of the reaction was 58 min.

2.3.1.3 High Resolution Melt (HRM) Data Analysis

Every PCR product of the SSD reaction was transferred into 20 µl Light Cycler capillaries (Roche). The products were melted in the HR-1 HRM appliance (Idaho Technology) at a rate of 0.3°C /s, with an initial temperature of 60°C, a final temperature of 90^0C and fluorescence data acquisition at 70-85°C. Finally, the data were evaluated using the HR-1 analysis tool custom software, with both derivative and difference plots produced after normalising and temperature shifting. The derivative and difference plots were visually reviewed with the purpose of isolating the mutants from the wild-type samples with a threshold of 4% difference in fluorescence as described by (Fadhil et al., 2010).

2.4 Construction of CD24 - pcDNA3.1 Plasmid

The pcDNA™3.1 Directional TOPO® Expression Kit (Invitrogen) was used to clone a blunt-end PCR product into pcDNA™3.1D/V5-His-TOPO® according to the manufacturer's instructions. The expression construct was manufactured by our previous group member Dr Mohamed Ahmed. The expression vector (5.5

kb) was principally designed to enable rapid directional cloning of blunt-end PCR products for their expression in mammalian cells. The plasmid map is shown in Appendices Chapter (Figure 9-17). The amplification primers for CD24 were manually designed in our lab (Table 2-2). The forward PCR primer was designed to include CACC bases at the 5′ end and before the ATG start codon to facilitate directional cloning. This enabled the primer to ligate with the overhang sequence GTGG in pcDNA™3.1D/V5-His-TOPO®. The reverse PCR primer was designed to not be complementary to the overhang sequence GTGG at the 5′ end with the aim of decreasing the possibility of ORF cloning in the opposite alignment. The amplification primer pair were synthesised by MWG-Biotech AG (UK) and diluted to give a working concentration of 100 pmol/µl.

Table 2-2. The PCR primers designed for CD24

PCR primer	Sequence
Forward	5’ CACCATGGGCAGAGCAATGGTGG 3’
Reverse	5’ TTAAGAGTAGAGATGCAGAAGAG 3’

2.4.1 Amplification of the CD24 Coding Sequence

The blunt end PCR product starts with the CACC at the 5’ end and was amplified using the designed primers in 50 µl PCR reactions using the Pfu DNA Polymerase (Promega) in a GeneAmp PCR system 2400 thermocycler (Perkin Elmer). The PCR reaction constituents are listed in Table 2-3 and cycling conditions were: 1 cycle of 95^0C for 2 min (initial denaturation), 35 cycles of 95^0C for 1 min (denaturation), 57^0C for 30 s (annealing) and 72^0C for 2 min (extension) and a final cycle at 72^0C for 10 min (final extension). PCR products

were evaluated using a 2% agarose gel to check for a single discrete band appropriate for direct cloning into pcDNA™3.1 Directional TOPO®.

Table 2-3. PCR reaction set up for amplification of the CD24 PCR product.

Component	1x	4 x	Final conc.
Pfu DNA Polymerase 10X Buffer with MgSO4	5 µl	20 µl	1X
dNTP mix, 10mM	1 µl	4 µl	200 µM
Forward primer	2 µl	8 µl	250 nM
Reverse primer	2 µl	8 µl	250 nM
DNA (CD24Image clone) 2 ng/µl	10 µl	Added separately	20 ng/reaction
Pfu DNA Polymerase (2-3 u/µl)	1 µl	4 µl	
Nuclease-Free Water	29 µl	116 µl	

2.5 Plasmid Transformation

The heat shock method was used to transform the plasmids into gold efficiency competent *E.coli* cells (Invitrogen). Briefly, 5 µl of each plasmid (pcDNA3.1 empty vector and pcDNA3.1-CD24 plasmids) were placed into a small vial containing 50 µl of *E.coli* competent cells. The mixture was placed on ice for 30 min, heat shocked for 45 s at 42^0C and cooled on ice for 5 min. Following this, 950 µl of warmed SOC outgrowth medium (2% Trypton, 0.5% yeast extract, 0.4% glucose, 10 mM NaCl, 2.5 mM KCl, 10 nM MgCl and 10 mM $mgSO_4$) (New England BioLabs, UK) were added to the mixture. The vials were then closed tightly and incubated on a shaker (200 rpm) for 1 hour at 37^0C. Cells were then centrifuged at maximum speed for 1 min, 800 µl of supernatant was discarded and the remainder (150 µl) was mixed with the cell pellet and aseptically plated on 10 cm petri dishes (Costar, UK) containing 25 ml Luria Broth (LB) agar (Sigma) and 25 µg/ml ampicillin (Sigma). The plates were

incubated overnight in an inverted position at 37^0C. The next day, one colony was carefully picked up using a sterile pipette tip and grown in a 5 ml LB broth medium supplemented with 5 µg/ml of ampicillin overnight at 37^0C on a shaker (200 rpm). For further growth, 5 ml of the mixture was transferred into a large sterile flask containing 100 ml LB medium and 100 µg/ml ampicillin, and incubated overnight at 37^0C on a shaker (200 rpm).

2.5.1 Plasmid Purification

The GenElute™ HP Plasmid Midiprep Kit (Sigma) was used according to the manufacturer's instructions. Briefly, following the plasmid transformation step, the bacterial culture was centrifuged at 5,000 g for 10 min and each pellet was re-suspended in 4 ml of chilled Resuspension/RNase A Solution and vortexed. Lysis solution (4 ml) was then added and mixed gently. After incubation for 5 min, the cells were re-suspended with 4 ml of chilled neutralisation solution, then with 3 ml of binding solution. After placing the GenElute™ Midiprep binding column onto the vacuum, 4 ml of column preparation solution was added to the column and allowed to pass through, then centrifuged at 3,000 g for 2 min. The eluate was discarded and the cell lysate was expelled into the Midi-Prep column, centrifuged at 3,000 g for 2 min and the eluate discarded. This step was repeated for the remaining cell lysate. The column was then washed, firstly with 4 ml of Solution 1 and then with 4 ml of Wash Solution 2 and centrifuged at 3,000 g for 2 min after each wash. The binding column was transferred to a new collection tube and the plasmid DNA eluted with 1 ml of elution solution (centrifugation at 3,000 g for 5 min). Finally, the concentration for each plasmid DNA was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific).

2.5.2 Quantification of DNA Plasmids, RNA and DNA

To assess the quality of and measure the concentration of DNA plasmids, extracted DNA or RNA samples, the spectrophotometer (NanoDrop™ 1000 Spectrophotometer) was firstly blanked with either the elution buffer or RNase free water. The absorbance of each sample was read at 260 nm and 280 nm. The measurement at A260 provides the quantity of the sample or DNA plasmid, and the ratio A260/A280 provides an estimation of the purity. A ratio greater than 1.8 was accepted as "pure" for DNA, while a ratio of 2.0 was accepted as "pure" for RNA. A ratio markedly different indicates the presence of protein, phenol or other contaminants within the sample. After plasmid quantification, the integrity of the plasmids was checked and 0.5 µg of each DNA plasmid in a volume of 10 µl plus DNA loading buffer (Promega) were run along with 5 µl of 1 kb DNA ladder (Promega) on a 1% agarose gel comprising SYBR Safe for 50 min at 100 V. The products were visualized using a gel documentation viewing system.

To check the presence of the CD24 insert in the construct, the plasmid DNA was evaluated by PCR, the primers used were the CD24 primers that were used for the amplification reaction of the coding sequences shown earlier. To confirm the precise orientation of the insert, a combination of the BGH reverse primer (included in the kit) and a CD24 forward primer were used. To further check the precise orientation of the insert and to eliminate mutation in the CD24 coding sequence, 2 of the plasmid DNA samples were sent to direct sequencing reaction. The T7 (forward) and BGH (Reverse) Vector primers were used for the sequencing. The sequencing data were analysed using the Chromaslite software available at: http://www.technelysium.com.au/chromas_lite.html.

2.6 Cell Transfection

Cell transfection was optimized considering multiple factors including cell passage number, confluence in T75 cell culture flask or 6-well plates, volume of plasmids transfected into the cell, final concentration of siRNA added to the reaction, volume of Lipofectamine 2000 (Invitrogen) transfection reagent and the amount of GIBCO™ Opti-MEM I reduced-serum medium (1X) (Invitrogen) as a diluent added to the reaction. Western blotting and flow cytometry techniques were used to evaluate the above conditions for different experiments. The optimal conditions for transfection of DNA plasmids were a volume of 4 µg of pcDNA3.1 empty vector and pcDNA3.1-CD24 plasmids together with 10 µl Lipofectamine 2000 in a total volume of 1.5 ml of Opti-MEM media. The optimal conditions for cell transfection of Cten and green fluorescent protein (GFP, the pEGFP-C1 plasmid (CLONETECH laboratories Inc.) inserted with Cten was kindly donated by Prof Su Hao Lo) constructs were 5 µg of plasmid DNA together with 10 µl Lipofectamine 2000. The conditions for cell transfection with siRNAs were like the conditions of transfecting cell with DNA plasmids, with the exception that the efficiency of the transfection for both non-targeting siRNA (luciferase) and siRNA of the gene of interest was considered optimal at a final concentration of 100 pmol for CD24 and Cten. The optimisation was performed using different volumes of Lipofectamine 2000 (5 to 20 µl) incubated with 50-100 pmol of siRNA duplexes. The transfection efficiency was evaluated using Western blot test.

2.6.1 Forced Gene Expression

The transfection process was conducted in accordance with manufacturer's instructions using the optimised conditions. Approximately 2 x 10^5 cells were seeded in each well of a 6-well plate (Costar) containing 2 ml of media (without antibiotics) and then incubated for 24 hrs at 37^0C. When the cells reached 60-70% confluence, transfection with empty vector (control) and DNA plasmids was performed. Prior to the transfection, the full media was replaced with Opti-MEM (1X) and incubated for 2 hrs at 37°C with 5% CO_2. The optimised volume of Lipofectamine 2000 was then added to 250 µl of Opti-MEM I reduced-serum medium (1X) and incubated for 5 min. The optimal plasmid concentration was also added to 250 µl of Opti-MEM (1X) in corresponding tubes. Next, the diluted Lipofectamine 2000 was added to the diluted plasmids to give a total volume of 500 µl. The mixture was incubated for 20 min at room temperature, added dropwise to the cells and incubated at 37°C. Six hrs later, the Opti-MEM media was replaced with fresh media supplemented with 10% FBS and the cells were harvested 24 hrs post transfection.

2.6.2 Gene Knockdown

The gene knockdown procedure was performed according to the protocol used for the plasmid transfection. Validated siRNA duplexes (Invitrogen) were used for knocking down the gene of interest. The siRNAs (20 nM) were dissolved in 1 ml of RNAse free water to make a stock solution of 20 µM. All the transfections were performed in 6-well plates in triplicate, with approximately 2 × 10^5cells seeded into each well and allowed to grow to 40-50% confluence. For cell transfection with CD24 siRNA, 100 pmol (10 µl) of the siRNA and 10 µl of

Lipofectamine 2000 for each well were individually diluted in 250 µl of Opti-MEM media as described above for the transfection optimization protocol. For Cten knockdown, the optimized concentration of siRNA was 100 pmol and the optimised volume of Lipofectamine 2000 was 5 µl. Non-targeting siRNA (luciferase) was used as a control and transfected at the same concentration as the target siRNA. The latter steps of transfecting siRNA to the cells were performed as described in the gene forced expression method except that the cells were harvested 48-72 hrs post transfection. The siRNAs used are listed in Table 2-4.

Table 2-4. The siRNAs sequences

Gene	Sequences
CD24 NM_013230	**L** ACCACGCUUAGUUAUGACCUACGAA **R** UUCGUAGGUCAUAACUAAGCGUGGU
Cten NM_032865	**L** AAGAGUAACUGUACCACGAGACCCG **R** UUCUCAUUGACAUGGUGCUCUGGGC
Universal non-targeting siRNA (Luciferase specific)	**L** CAGUGUAGUAGUCGUUUC **R** GAAACGACUACUACACUG

2.6.3 Co-transfection

Occasionally, a dual approach of forced expression of a specific molecule and a knockdown of another one in the same experiment is necessary to determine a potential association between these molecules. For each condition, cells were transfected with optimal DNA plasmids mixed either with an optimal concentration of luciferase as a universal non-targeting siRNA or an specific siRNA as described in the forced expression and knockdown protocols. The related experiments were performed 48 hrs post transfection and a potential

association between the two molecules was assessed using Western blotting and/or flow cytometry.

2.7 Western Blotting

2.7.1 SDS-PAGE

NuPAGE® gradient (7-12%) gels were used for all the Western blot experiments. Briefly, the gel was placed into a 1X NuPAGE® MOPS SDS running buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7) (Invitrogen) in the electrophoresis unit. Fifty or 25 μg of each protein were added to the 4X NuPAGE® LDS sample buffer (pH 8.4) (Invitrogen) containing 5% β-mercaptoethanol (Sigma). The proteins were then heated at 90°C for 5 min in order to denature the protein, followed by cooling on ice for 5 min. Afterwards, the proteins were centrifuged for 1 min and carefully loaded into the gel wells. The protein marker, Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad), was loaded into the first well and the gel was run for 90 min at 125 V.

2.7.2 Transfer of Proteins from SDS-PAGE

A semi-dry system was used for protein transfer. Briefly, the polyvinylidene difluoride (PVDF) membrane (GE healthcare Life Sciences, UK) was initially activated using 20% methanol for 10 s and then hydrated in transfer buffer before placing it into the diluted NuPAGE® transfer buffer (20X) (Invitrogen). Two pieces of filter paper (for each gel) were cut to the same size as the gel and wetted with the transfer buffer. The top-left part of the gel and the membrane were cut to maintain the correct orientation. The sandwich was made as follows: the PVDF membrane was placed over the filter paper, the gel was placed over

the membrane, the second filter paper was placed over the gel and the air bubbles were removed using a roller. Finally, the sandwich was placed in the semi-dry transfer apparatus (Bio-Rad) and run at 25 V for 30 min.

2.7.3 Protein Immuno-Detection

After the protein transfer step, the membrane was immersed in either a 5% milk solution or BSA dissolved in 0.1% Tween 20 PBS on a rocking platform for 1 hr at room temperature to block non-specific binding sites. The membrane was then treated with an appropriately diluted primary antibody (Table 2-5) and rocked overnight at 4°C. The membrane was removed from the primary antibody and washed three times for 5 min each with Tris-buffered saline and Tween 20 (TBST). An appropriate horseradish peroxidase (HRP)-linked secondary antibody (anti-rabbit or anti-mouse) was prepared 5 min prior to the third wash, and then the membrane was incubated with the antibody on a rocking platform at room temperature for 1 hr. The membrane was washed twice with TBST, 5 min each, and a final wash with normal PBS for 5 min. The Amersham Enhanced Chemiluminescence (ECL) Prime Western Blotting Detection Reagent kit (GE Healthcare Life Sciences) was used for protein detection. The detection solutions were allowed to equilibrate to room temperature for 20 min before starting the detection. Solution A (luminol) was added to solution B (peroxide) in a ratio of 1:1 to form a working solution. The excess wash buffer was drained from the membrane and the membrane was placed in a suitable, clean container. The detection reagent was then placed onto the whole membrane followed by incubation in the dark at room temperature for 5 min. Finally, the membrane was placed with the protein side up in an X-ray film cassette and then the film (Kodak, UK) exposed for different periods of time,

followed by rinsing in developing, then in fixation solutions before washing in tap water. The C-DiGit® Blot Scanner (LI-COR Biotechnology, UK Ltd) was also used for protein-immunodetection and the Western blot images were analysed using Image Studio™ Software (LI-COR Biotechnology).

Table 2-5. Antibodies used for Western blotting.

Antibodies	MW kDa	Supplier	Primary Ab.	Secondary Ab.	Ab. type
Anti-CD24 SWA11	30-70	gifted from Prof. P. Altevogt, Germany	1:2,000 5% MILK	1:5,000 5% MILK	Anti-mouse
Anti- Cten	78	Sigma # WH0084951M1	1:5,000 5% MILK	1:10,000 5% MILK	Anti-mouse
Anti-ILK	50	Cell signalling #3862S	1:1,000 5% BSA	1:5,000 5% BSA	Anti-rabbit
Anti-FAK	125	Cell signalling #3285S	1:1,000 5% BSA	1:5,000 5% BSA/TBST	Anti-rabbit
Anti-p-FAK	125	Cell signalling #3283S	1:1,000 5% BSA	1:5,000 5% BSA/TBST	Anti-rabbit
Anti- N- cadherin	140	Abcam # ab98952	1:1,000 5% BSA	1:5,000 5% BSA	Anti-mouse
Anti- E- cadherin	135	Cell signalling #3195S	1:1,000 5% BSA	1:5,000 5% BSA	Anti-rabbit
Anti- Snail	29	Cell signalling #3879S	1:5,000 5% BSA	1:5000 5% BSA	Anti-mouse
Anti-Src	60	Cell signalling #2108S	1:1,000 5% BSA	1:5,000 5%BSA	Anti-rabbit
Anti-β- actin	42	Abcam #ab8229	1:50,000 5% MILK	1:50,000 5% MILK	Anti-mouse
Anti-Vimentin	57	Cell signalling #5741S	1:2,000 5% BSA	1:15,000 5% BSA	Anti-rabbit
Anti-Vinculin	145	Cell signalling #4650S	1:1,000 5% BSA	1:10,000 5% BSA	Anti-rabbit
Anti-Paxillin	68	Life Span Bio #LSC49487	1:1,000 5% BSA	1:5,000 5% BSA	Anti-rabbit
Anti-β-catenin	92	Enzo #ALX-804-060-C100	1:1,000 5% MILK	1:5,000 5% MILK	Anti-mouse
Anti-kras	22	Cell signalling #3965S	1:250 5% MILK	1:5,000 5% BSA/TBST	Anti-mouse
Anti-Bcl-2	26	Abcam # ab59348	1:500 5% MILK	1:1,000 5% BSA/TBST	Anti-mouse
Anti-Bcl-xL	26	Abcam # ab32370	1:100 5% MILK	1:1,000 5% BSA/TBST	Anti-rabbit
Anti-EGFR	175	Abcam#ab52894	1:1,000 5% BSA	1:2,000 5% BSA/TBST	Anti-rabbit
Anti-p-EGFR	137	Abcam #ab40815	1:10,000 5% BSA	1:2,000 5% BSA/TBST	Anti-rabbit
Anti-Akt (pan)	60	Cell signalling, #2920S	1:2000 5% BSA	1:2000 5% BSA/TBST	Anti-rabbit
Anti-P-Akt (Ser473)	60	Cell signalling #4060S	1:2000 5% BSA	1:2000 5% BSA/TBST	Anti-rabbit
Anti-P-Akt (Thr308)	60	Cell signalling # 2965S	1:2000 5% BSA	1:2000 5% BSA/TBST	Anti-rabbit
p-eNOS (Ser1177)	140	Sigma	1:1000 5% BSA	1:2000 5% BSA/TBST	Anti-rabbit
p-CREB	43	Sigma	1:1000 5% BSA	1:2000 5% BSA/TBST	Anti-rabbit
Anti-Tubulin	50	Abcam#ab6160	1:2000 5% MILK	1:5,000 5% MILK	Anti-mouse
Anti-lamin	68	Abcam#ab8982	1:5000 5% MILK	1:1,000 5% MILK	Anti-mouse

2.8 Co-Immunoprecipitation (Co-IP) Assay

The purpose of this experiment was to identify a possible interaction between specific molecules (e.g. CD24 and Cten), as it is well-known that these molecules localise in the cellular cytoplasm. After protein quantification, a cell lysate pre-clearing step was performed as follows; 20 µl of Protein G Plus/Protein A Agarose Suspension-Beads (Calbiochem, UK) were added to 1 ml of the protein lysate and placed on a rocking platform at 4°C for 30 min. The mixture was then centrifuged at maximum speed for 10 min at 4°C. The supernatant was extracted and used for Co-IP. Eppendorf tubes (1.5 ml) were labelled ("input", "control", "CD24" and "Cten") and 300 µg of protein lysate were added to each tube, except for the input tubes, to which just 50 µg of the lysate were added. Two microliters of anti-CD24 clone SWA11 (gifted from Prof. P. Altevogt, Germany) and anti-Cten (Sigma) antibodies were added to the corresponding tubes and incubated overnight on a roller at 4°C. Then, 30 µl of beads were placed into each tube, except for the input tubes, before vortexing and incubation overnight at 4°C on a rotator. On the third day, the tubes were spun for 5 min at 13,000 rpm at 4°C and the beads were washed 3X with PBS for 5 min each. After that, 20 µl of NuPAGE® loading 4X buffer supplemented with 5 µl of β-mercaptoethanol was added to each tube and heated for 5 min at 90°C, placed on ice for 5 min, centrifuged for 1 min at 13,000 rpm and finally, run on a SDS-PAGE.

2.9 Functional Assays

2.9.1 PrestoBlue Proliferation Assay

PrestoBlue® Cell Viability Reagent (Invitrogen) was used to quantify cell proliferation after gene manipulation. This technique is very convenient for evaluating cell proliferation since the dye used is very sensitive and not toxic to cells. In addition, it provides the opportunity to obtain more accurate data as the same cells are used for several days and minimises potential errors. Briefly, the required number of cells for seeding (usually 5 x 10^4) was counted using an automated cell counter and loaded into each well of 24-well plate, incubated either for 8 hrs or overnight at 37°C. A master mix of 10% PrestoBlue® Cell Viability Reagent was prepared with full media in a ratio of 1:10 and after aspirating the media, 400 µl of the master mix was added to each well of the 24-well plate in triplicate. The plate was then covered with foil (the dye is sensitive to light) and incubated at 37°C for 1 hr. Then, 100 µl from each well was taken out and loaded in triplicate into a 96-well microplate, while 100 µl of the master mix of 10% PrestoBlue® was also added into one well as a blank. The fluorescence units in each well were read using a FLUOstar OPTIMA (BMG, Lab Tech) using different gains (500, 1000, and 1500). The readings were taken at an excitation 550 nm and emission 620 nm band widths at 1000. Once the PrestoBlue mix had been taken from the cells, the medium containing the reagent was aspirated and 1 ml of the full media was added to each well to allow the cells to continue to grow until the next measurement of cell number. This scenario was repeated approximately every 24-hrs until the assay was terminated. Cells were seeded in triplicate for each experimental condition.

2.9.2 Migration Assays

2.9.2.1 Transwell Migration Assay

The assay was performed for the purpose of investigating the effect of manipulating the gene of interest on cancer cell motility. Briefly, 24 to 48 hrs post transfection with the vector / siRNA of interest, the migration assay was performed using a 6.5 mm Transwell with 0.4 μm pore polyester membrane insert (Costar). Two hrs prior to cell harvesting, the transwells were immersed in 600 μl of DMEM supplemented with 10% FBS in the upper wells of a 24-well plate and incubated at 37°C to activate the membranes. The cells were then washed twice with PBS, trypsinized and full media (500 μl) was added to each well of the 6-well plate, followed by transfer of the cell suspension into sterile Eppendorf tubes and centrifugation for 5 min at 1000 rpm. The supernatant was then discarded and the pellet was re-suspended in 1 ml of fresh full media. Cell counting was performed (as described above) and 5×10^4 to 1×10^5 cells were mixed in the appropriate volume of full media and seeded in each well in triplicate. Media supplemented with 20% FBS (600 μl in each well) was added into the lower chambers. The tissue-culture treated membranes were transferred into wells and 100 μl of the cell suspension mixture was added into the upper chambers. The cells were then allowed to migrate at 37°C and 5% CO_2 in humid conditions for 24 to 48 hrs. Cells were seeded in triplicate for each experimental condition.

Three different approaches of cell counting were used in cell migration and invasion assays. The standard method involved viewing the migrated/invaded cells under an inverted microscope at a magnification of 10x and counting in different fields of view to get an average sum of cells that have migrated through

the membrane. The second method was to count cells directly from the transwells membrane as follows: the membrane was washed twice with PBS, fixed with 10% methanol for 30 min and then washed twice with PBS. The cells were removed from the upper part of the membranes using cotton swabs, trypsinized with trypsin-EDTA for 10 min, re-suspended with 1 ml media supplemented with 10% FBS and the cells were counted using the automated cell counter. The third method counted cells that have migrated through the membrane toward the chemo-attractant and attached on the underside of the membrane after removing the cells from the upper part of the membranes using cotton swabs and trypsinizing them with trypsin-EDTA for 10 min. The cells were re-suspended with 1 ml of media and counted using the automated cell counter. This method was used often as it is easy, accurate when matched with the first method and less-time consuming (Appendices, Figure 9-20).

2.9.2.2 Wound Healing Assay

Cells grown in 6-well plates were transfected with appropriate DNA plasmids and then allowed grown to 100% confluence. After marking the back of the plates with transverse lines, three scratches/wounds were made on the surface of the 6-well plates using a sterile 200 µl tip. The cells were then washed twice with PBS to get rid of the cells that came off. Two millilitres of media supplemented with 10% FBS was added to the wells and immediately photos (T = 0) were taken just above and below the marked lines. Approximately 24 hrs later, photos (T = 24) were taken at the same position as T = 0. The photos (T = 0 and T = 24) were then analysed by T-Scratch (CSElab) software using the following formula: area of migration = 100 x (open wound area at 0 hr –open wound area at 24 hrs). Cells were seeded in triplicate for each condition.

2.9.3 Matrigel Invasion Assay

The assay was performed using the polycarbonate membrane with a thin layer of growth factor free Matrigel (BD Bioscience, USA). Matrigel solution (8 mg/ml) was permitted to melt at 4°C and diluted with serum and antibiotic-free cold media (1:2). The mixture was then incubated overnight at 4°C. An appropriate volume of the diluted Matrigel was cautiously added to the polycarbonate membrane and allowed to solidify at 37°C and 5% CO_2 in the incubator for 4 hrs. Thereafter, 2 x 10^5 of cell suspension was applied over the Matrigel layer in the upper chamber of the transwell membrane after placing 600 µl media containing 20% FBS in the outer well of the 24-well plate. The plates were incubated for 48 hrs (37°C, 5% CO_2) and the cells invading the lower chamber were counted using the automated cell counter. Cells were seeded in triplicate for each experimental condition.

2.9.4 Soft Agar Colony Formation Assay

Two layers were prepared for the colony formation assay, base layer and top layer. Both 1% base agar layer and 0.7% top agarose layer were prepared under sterile conditions with cell culture grade H_2O heated in the microwave to dissolve and cooled in the water bath to 40°C. DMEM (2X) supplemented with 20% FBS and penicillin/streptomycin was warmed at 40°C in a water bath for 30 min and equivalent volumes of these solutions were mixed up to give 0.5% agar and DMEM (1X) with 10% FBS. One millilitre of the mixture was placed in each well of a 6 well-plate for each treatment condition in triplicate, including a blank and then left to solidify. Cells for each condition were trypsinized and diluted to 100,000 cells/ml. The cell suspension (0.1 ml) was transferred to a

sterilised tube containing 2 ml of DMEM (2X) and 2 ml of 0.7% agarose, mixed gently and then, 1 ml of the mixture was placed into each well (2,500 cells per well). The plates were incubated at 37°C for 21 days and cells fed with 0.5 ml DMEM (1X) every 3 days. Finally, the plates were fixed and stained with 0.5 ml of 0.005% crystal violet (Sigma) mixed with 4% formaldehyde solution (Sigma) for 1 hr and colonies counted under the light microscope.

2.9.5 Cell Treatments

2.9.5.1 Inhibition of PI3K

Appropriate cancer cell lines were treated with either the PI3K inhibitor LY294002 (10 μM, Cell Signalling) at a 20 ng/ml concentration or CD24 siRNA, or both combined. Controls comprised DMSO and non-targeting control siRNA, respectively.

2.9.5.2 Stimulation with Epidermal Growth Factor (EGF) and Inhibition of Epidermal Growth Factor Receptor (EGFR)

For stimulating with EGF, cells were treated with recombinant-EGF (Thermo Fisher Scientific) to activate signalling through the EGFR. The seeded cells in the 6-well plate were permitted to adhere for 4 hrs, then incubated for 24 hrs in serum free DMEM (supplemented with penicillin/streptomycin) prior to stimulation. At 60-70% confluency, cells were treated with different concentrations (10-100 ng/ml) of recombinant EGF (Thermo Fisher Scientific) under serum free conditions. After 24 hrs incubation, cells were harvested. For inhibition of EGFR signalling, cells were treated with PD153035 (Abcam, UK) at a final concentration of 20 μM 24 hrs prior to further analysis. The cells treated with DMSO as a control.

2.9.5.3 Cycloheximide Assay

The cycloheximide assay is used to measure protein stability. Cycloheximide (CHX) is an inhibitor of protein translation and, after treatment with CHX, the levels of a protein can be monitored over time to evaluate levels of protein stability. The CHX stock solution was purchased from (Sigma) and prepared with media supplemented with 10% FBS to give a final concentration of 100 µg/ml. Initially, the assay was performed with a 4 hr interval for 24 hrs as CHX might cause cytotoxicity to cells. Different cancer cell lines were also used with different concentrations of CHX to determine the conditions in which the CHX reagent works most efficiently. Cells were treated with CHX and approximately 24 hrs post transfection, the media in the 6-well plate was replaced with media containing an appropriate concentration of CHX (10-100 µg/ml) and incubated (37°C, 5% CO_2) for different time points dependent upon the half-life of the protein. Next, cells were washed with PBS, harvested and then treated with RIPA lysis buffer at the same point for all treatment groups for protein extraction and quantification. Finally, the lysates were aliquoted in new Eppendorf tubes for storage at -20°C and Western blotting was performed.

2.9.6 RNA Extraction

Total RNA extraction was performed using GenElute Mammalian Total RNA Mini-prep kit (Sigma) according to the manufacturer's protocol. Attached cells were lysed directly in wells after removing the culture medium and washing with twice with PBS. Briefly, a volume of 250 µl lysis buffer supplemented with 1% 2-mercaptoethanol (2-ME) was added to each well (containing approximately 5 x 10^6 cells) in 6 well-plates. To remove cellular debris and shear DNA, the lysate was homogenised in a filtration column at 13,000 rpm for 2 min, followed by

the addition of 250 µl of 70% ethanol to the lysate and mixed thoroughly. After this, each sample was transferred to a binding column, centrifuged at 13,000 rpm for 15 s and the flow through was discarded. After spinning, the binding column was placed back into the collection tube and 250 µl of wash 1 buffer was added to the column, centrifuged at 13,000 rpm for 15 s, the flow through was discarded and the collection tube replaced with a new one. For on-column DNase I digestion, a mastermix (10 µl of DNase I and 70 µl buffer per column) was prepared and mixed gently. Then, 80 µl of the mixture was added directly to the column, followed by incubation at room temperature for 15 min. A second wash with wash 1 buffer was performed (250 µl, centrifuged at 13,000 rpm for 15 s), the flow through was discarded and the collection tube replaced. Wash 2 buffer containing ethanol (500 µl) was added to the column, centrifuged at 13,000 rpm for 15 s, the flow through was discarded and the collection tube replaced before a second wash with wash 2 buffer (500 µl, centrifuged at 13,000 rpm for 2 min). The RNA was eluted into a new collection tube with 50 µl RNase free water added directly to the middle of the column and centrifugation at 13,000 rpm for 1 min. Finally, the RNA was quantified using the Nanodrop.

2.9.7 DNA Extraction

GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) was used to extract DNA. Briefly, cells were dissociated from the 6-well plate using trypsin-EDTA and then transferred to collection tubes. The pellet of 5 x 10^6 cells was made by spinning the samples for 5 min at 1500 rpm and washing twice with PBS to remove the culture medium. The pellet was re-suspended thoroughly in 200 µl of resuspension solution. To obtain RNA-free genomic DNA, 20 µl of RNase A Solution was added to each lysate and incubated for 2 min at room temperature.

Next, 20 µl of the Proteinase K solution was added to each sample, followed by 200 µl of lysis solution C, vortexed thoroughly for 15 s, and incubated at 70°C for 10 min. The column preparation solution (500 µl) was added to each pre-assembled GenElute Miniprep binding column, centrifuged at maximum speed for 1 min and the flow-through liquid discarded. A volume of 200 µl of absolute ethanol was added to the lysate, mixed thoroughly for 10 s and centrifuged at maximum speed for 1 min. The collection tube was discarded and the binding column placed in a new 2 ml collection tube. Wash solution (500 µl) was added to the binding column, centrifuged for 1 min at maximum speed and the collection tube containing the flow-through liquid discarded. The binding column was placed in a new collection tube and washed with 500 µl of wash solution, before centrifugation for 3 min at maximum speed to dry the binding column. An additional spin was performed for 1 min at maximum speed to completely remove the ethanol. The binding column was placed in a new 2 ml collection tube, 200 µl of the elution solution was added directly to the centre of the binding column and incubated for 5 min at room temperature before centrifugation for 1 min at maximum speed to elute the DNA. The DNA was quantified using the Nanodrop instrument.

2.9.8 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Initially, reverse transcription of RNA was performed to acquire complementary DNA (cDNA). For cDNA synthesis, 2 µg of sample RNA and 1 µl of random hexamers (Thermo Fisher Scientific) were prepared with nuclease free water to give a total volume of 15 µl and incubated at 70°C for 10 min as the initial denaturation step. The samples were then cooled on ice for 5 min and master mix was prepared in a 1.5 ml Eppendorf tube by adding 1 µl of Moloney Murine

Leukaemia Virus Reverse Transcriptase enzyme (M-MLV RT; Invitrogen), 2.5 µl (50 mM) of dithiothreitol (DTT; Invitrogen) and 1.5 µl of deoxyribonucleotide triphosphate (dNTP). In RT negative samples (RT-), water was added instead of the reverse transcriptase enzyme. The mastermix was mixed gently, centrifuged and 10 µl was added to each RNA sample (a final volume of 25 µl) and incubated at 37˚C for 1 hour followed by incubation at 95˚C for 10 min. The cDNA was diluted 1:10 with nuclease free water before gene quantification.

2.9.8.1 Designing the qPCR Primers

Primers for qPCR were designed using Primer 3 software (developed by Steve Rozen and Helen J. Skaletsky) available on-line at http://frodo.wi.mit.edu/ (see Table 2-6). The primer sequences were then analysed with the Amplifix program (available online at http://ifrjr.nord.univ-mrs.fr/AmplifX-Home-) for further characteristics: recognition of the primers for the gene of interest, possibility of primers forming dimers, and/or hairpins. Ultimately, all sequences were submitted to the *Blast* software, available on the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov) for the purpose of evaluating gene specificity. The primers were synthesized by Eurofins (UK).

Table 2-6. The qPCR primer sequences

Reference	Primer sequence	Annealing Temperature (^{o}C)
HPRT	Forward: TCCCCTGTTGACTGGTCATT Reverse: AAATTCTTTGCTGACCTGCTG	60
Cten	Forward: TTGGAAGCAGCCAGTC Reverse: GAGGAAGAGTTGGCTGGA	55
CD24	Forward: ACCCACGCAGATTTATTCCA Reverse: CCTTGGTGGTGGCATTAGTT	59

2.9.8.2 Quantitative Real-Time PCR

At first, the annealing temperature was optimised for each primer set using gradient PCR. The real-time PCR reaction included Go Taq DNA Polymerase (Promega), the forward and reverse primers (250 nM) and 10% cDNA in a total volume of 10 µl. The reaction cycle conditions were: 1 cycle of 95°C for 2 min, 40 cycles of 95°C for 30 sec, 60°C +/- 10°C for 30 sec and 72°C for 30 sec, and 72°C for 10 min using the thermal cycler (MWG Biotech Inc.). Once the annealing temperature was optimised, the efficiency of the reaction was evaluated using a standard curve. The qPCR was conducted using the 7500 Fast Real-Time PCR System (Applied Biosystems) with the following reaction cycling: 95°C for 2 min, 40 cycles of 95°C for 3 sec and optimised annealing temperature for 30 sec). The next step was the melting curve from 60-95°C and reactions with an efficiency of 90-110% were considered acceptable. After optimisation, qPCR was performed in a final reaction volume of 25 µl using the 7500 Fast Real-Time PCR System and cycling conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 3 sec and optimised annealing temperature for 30 sec. The standard curve was prepared from a serial dilution of neat cDNA. No template control (NTC) without any cDNA and no RT (RT-) control without reverse transcriptase enzyme were included in every experiment. Each sample was run in triplicate and amplification was normalized to the housekeeping gene Homo Sapiens hypoxanthine phosphoribosyl transferase 1 (HPRT1). The Delta delta CT method (2-ΔΔCt) method was used for gene quantification.

2.9.9 Agarose Gel Electrophoresis

Agarose gel (2%) was prepared by dissolving 2 g of agarose (Gibco-BRLife Technologies, USA) in 100 ml of 1% Tris Borate EDTA (TBE) buffer (Sigma) in a sterilized conical flask and then heating for 1 min in a microwave. Next, the gel was cooled to room temperature under running tap water. A volume of 10 µl of SYBR Safe DNA fluorescent dye (1000X; Sigma) was added to the gel, transferred into the gel running plate and a suitable size comb was inserted. The gel was then left for about 20 min in the dark at room temperature to solidify. A total volume of 12 µl (10 µl of DNA sample mixed with 2 µl loading dye) was loaded into the wells and 100 bp DNA ladder (New England BioLabs, UK) was loaded to show the size of the samples. The gel was run at 90 V for 30 min using 1X TBE running buffer. PCR products were visualized using an ultraviolet trans-illuminator (UVP Inc., USA).

2.10 Flow Cytometry

Flow cytometry was used to detect the efficiency of the CD24-plasmid or CD24 siRNA transfection and compared to control (EV or luciferase) to attain the highest efficiency in cell lines. Briefly, cells in the 6-well plate were washed twice with PBS, followed by adding the cell dissociation buffer (non-enzymatic cell dissociation solution, Sigma). Cells were then transferred to a 20 ml universal tube and centrifuged for 3 min at 1000 rpm. Next, cells were re-suspended with 500 µl of PBS, transferred into fluorescence-activated cell sorting (FACS) tubes and observed using the Coulter Altra Flow cytometer (Beckman Coulter) with a GFP filter. The data was analysed using Weasel software v3.0.2.

2.11 Immunohistochemistry

Prepared CRC and NSCLC tissue microarray (TMAs) 4 μm sections were mounted on Superfrost slides (Surgipath, UK). The NovoLink™ Polymer Detection System was used for both set of TMAs. After labelling the slides, they were placed on a hotplate for 10 min at 60°C to enhance the adhesion of tissue sections to the slide surface. Once the sections had cooled, they were placed in an auto-stainer slide rack for dewaxing and rehydration (xylene 2 x 5 min, IMS 3 x 2 min, water 5 min). After washing the slides in a water bath, antigen epitope retrieval was induced using microwave treatment of sections (at full power) in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Slides were loaded into Shandon Sequenza Coverplates (Thermo Scientific) and then placed into Sequenza trays before rinsing in TBS (0.05 mol/l TrisHCl, 0.15 mol/l NaCl, pH 7.6). The activity of endogenous peroxidase was blocked by applying 100 μl of peroxidase block for 5 min. Sections were then washed twice with TBS (5 min), followed by incubation with 100 μl of protein block for 5 min. After 2 washes with TBS, 100 μl of primary antibody optimally diluted in Leica antibody diluent (RE7133) was applied to the sections and incubated for an hour. After 2 washes, 100 μl of post primary block was applied for 30 min, before 2 washes with TBS and application of 100 μl of Novolink Polymer for 30 min. After the sections were washed twice with TBS, they were treated with 100 μl of freshly prepared diaminobenzidine (DAB) working solution (1:20), followed by another wash. The sections were then counterstained with Novolink haematoxylin for 6 min. After rinsing in tap water for 5 min, sections were dehydrated in alcohol, cleared in xylene (GentaMedica, York, UK) and mounted with DPX mounting medium (BDH, UK). Negative and positive controls were also included.

The diluent buffer alone without the primary antibody formed the basis of negative controls. Positive controls included tissues from liver and lung known to express the antigen of interest with known reactivity to each antibody as suggested by the manufacturer's data sheet. The optimal staining conditions for each antibody were identified; variables including the dilution factor of the antibody, incubation time with the primary antibody and the nature of antigen retrieval method were optimised prior to IHC staining. Antibodies used in IHC staining are summarised in the Appendices chapter (Table 9-3).

2.12 Subcellular Protein Fractionation

The NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit (Thermo scientific, 78833) was used to separate and prepare cytoplasmic and nuclear extracts from cultivated cells. For cell culture preparation, adherent cells were harvested with trypsin-EDTA and then centrifuged at 500 × *g* for 5 min. After washing with PBS, the cell pellet was re-suspended with PBS and ~1×10^6 cells were transferred to a 1.5mL Eppendorf tube, followed by centrifugation at 500 × *g* for 2-3 min. The supernatant was carefully removed using a pipette, leaving the cell pellet dried.

For cytoplasmic extraction, 200 µl ice-cold cytoplasmic extraction reagent I (CER I) was added to the cell pellet immediately after adding an appropriate volume of protease inhibitors to minimize reagent dilution. The tube was then vortexed on the highest setting for 15 sec to fully suspend the cell pellet, followed by incubation on ice for 10 min. Ice-cold CER II (11 µl) was added to the tube, which was vortexed for 5 sec, and incubated on ice for 1 min. Another vortex for 5 sec on the highest setting was carried out before centrifuging the

tube for 5 min at maximum speed (13,000 × g). The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube and placed on ice until use.

For nuclear extraction, the pellet produced after the centrifugation step, which contains nuclei, was suspended in 100µl ice-cold nuclear extraction reagent (NER) immediately after adding an appropriate volume of protease inhibitors, and vortexed on the highest setting for 15 sec. The tube was placed on ice and vortexed for 15 sec every 10 min, for a total of 40 min. Following centrifugation at maximum speed (13,000 × *g*) for 10 min, the supernatant (nuclear extract) fraction was transferred to a clean pre-chilled tube and placed on ice until use. Finally, the cytoplasmic and nuclear protein extracts were quantified and run on an SDS-PAGE gel.

2.13 Statistical Analysis

All functional study data was analysed using Graphpad Prism 6 software. The data were presented as the mean ± standard deviation (SD), in triplicates and from three independent experiments. Statistical tests for normally distributed data, the unpaired t-test (for data sets comprising 2 treatment groups) or the analysis of variance (ANOVA) (for more than 2 treatment groups) were applied. A p-value of <0.05 was considered significant.

For the IHC data, IBM SPSS Statistics v22.0 software was used for the analysis of TMA staining. The Chi-squared test (Inc. Fisher's exact test) and Pearson's test were used for association between categorical data. The median H-score (H-score is a method of assessing the level of immunoreactivity) was considered as the cut off to label tumours as having low, moderate or high marker expression.

3 INVESTIGATING THE REGULATORY ROLE OF CD24 IN CANCER

3.1 Introduction

CD24 is a cell adhesion molecule that has been found to have oncogenic features. It is able to promote the anchorage-independent growth of cancer cells (i.e. stemness), cell migration, invasion, and proliferation (Wang et al., 2010). These features are important in the development of metastatic tumour (Weichert et al., 2005a).

The metastatic process can be broken down into a number of steps such as migration of cells away from the main tumour mass and into blood / lymphatic vessels, exiting the vessels at the site of metastasis and growth at the new metastatic site. CD24 can, in theory at least, support each of these steps. For example, it is thought that CD24 can bind to P-selectin and this enables cancer cells to bind to the activated platelets and endothelial cells on the inner surface of blood vessels, thereby leading to "stasis" in the blood vessel followed by migration out of the vessel into a new site (as part of the metastasis process) (Baumann et al., 2005).

The localisation of CD24 in the lipid rafts supports the notion that this molecule may play a role in regulating a variety of cellular functions (Bowie et al., 2012). Previous studies have found that the entrance of CXCR4 and β1-integrins into this surface domain is regulated by CD24 (Runz et al., 2008, Schabath et al., 2006). This process may lead to integrin activation and it is significant for its function during cell adhesion, migration, and invasion (Mierke et al., 2011a). Various other CD24 influenced adhesion molecules have been acknowledged to work as factors of cancer cell invasion and metastasis formation (Mierke et al., 2011a, Mierke et al., 2011b). Likewise, CD24 has been shown to activate STAT3 and src, boosting cancer-promoting activities involved in stemness, cell

proliferation and metastasis (Bretz et al., 2012b). In addition, experiments performed *in vitro* and *in vivo* have also shown that cellular functions, including adhesion, proliferation, invasion, and tumour growth, were altered following the manipulation of CD24 (Bretz et al., 2012b, Wang et al., 2010, Farahani et al., 2014).

CD24 has been reported to be involved in up to 90% of gastrointestinal tumours (Naumov et al., 2014), including CRCs (Kraus et al., 2015), HCC (Yang et al., 2009), NSCLCs (Lee et al., 2010) and pancreatic ductal adenocarcinoma (Rao and Mohammed, 2015). We have previously shown that CD24 is associated with metastasis promoting activity in colorectal cancer cell lines. CD24 is also up-regulated in a variety of other types of epithelial cancer suggesting that it may represent a "common" pathway for metastasis. Thus, it was hypothesised that CD24 activity is critical for modulating cellular functions in different forms of cancer in a similar way to CRC. To undertake this hypothesis testing, functional studies in three cancer models were undertaken (and CRC data were validated). As models, we chose cancer of pancreas, lung and liver. These cancers which driver mutations in common with CRC and which, in common with the colon, arise from embryonic endoderm.

3.2 Results

The expression levels of CD24 in the four-cancer model mentioned above were manipulated using a dual approach of forced CD24 expression and CD24 knockdown, after which the changes which occurred in cell function were evaluated. Activities including cell migration, invasion, proliferation, and colony formation were evaluated using appropriate functional assays. To verify

the accuracy of our results and to validate our observations further, all relevant experiments were performed three separate times in triplicate, and the data from the four-cancer models were analysed to check for consistency within the four tested models.

3.2.1 Investigation of the Role of CD24 in Regulating Cell Functions in CRC

The purpose of the first set of conducted experiments was to determine whether forced expression of CD24 in cells with low or absent CD24 expression influences cellular functions, such as motility, invasion, and proliferation, in these cell lines. CD24 was forcibly expressed in the CRC cell lines, HCT116 (which shows very low expression of CD24) and RKO (which is negative for CD24 expression). Cells were transfected with the vector expressing CD24 and controls were transfected with the same vector but without the CD24 DNA present, called "Empty Vector" or EV. Expression of CD24 in all tested cell lines was confirmed by Western blot test (see Appendices, Figure 9-3).

Transwell migration, wound healing and Matrigel invasion assays were performed 48 hour post transfection of cell with the expression vector as described in Chapter 2. Both migration assays, i.e. Transwell and wound healing, were performed following overexpression of CD24 in HCT116 cell line and the data showed that the increased levels of CD24 resulted in a significant increase in cell motility ($p=0.0009$ and $p=0.030$, respectively; Figures 3-1A & 3-2A&B). Cell motility (by Transwell migration) was significantly increased following overexpression of CD24 in the RKO cell line ($p<0.0001$; Figure 3.1B). Similarly, this transfection showed a significant increase in cell invasion

through Matrigel in both cell lines (p=0.0003 and $p<0.0001$, respectively; Figure 3-3A&B).

The effect of CD24 on cell number was tested using the PrestoBlue proliferation assay. Forced expression of CD24 in HCT116 and RKO did not affect cell proliferation compared with EV (p=0.415 and p=0.484, respectively; Figure 3-4A&B). The lack of effect on cell number also demonstrated that the effects observed on motility were not likely to be artefacts due to differences in cell numbers.

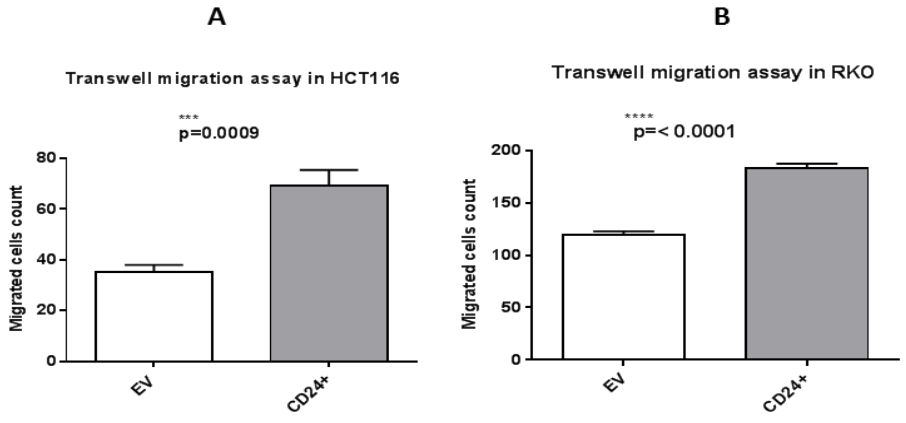


Figure 3-1. Transwell migration assay in CD24-negative CRC cell lines. The assay was performed following cell transfection with CD24+ and EV in the HCT116 and RKO cell lines. In a comparison to the control, forced expression of CD24 in both cell lines resulted in a significant increase in cell motility ($p<0.05$). The assay was performed in triplicate and the data were analysed using Prism Pad 6 software (unpaired t-test).

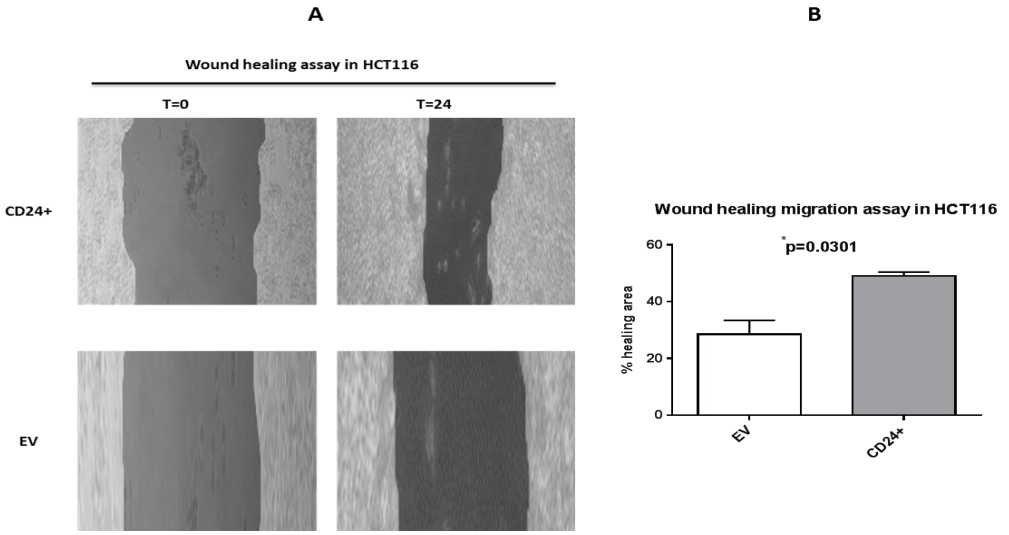


Figure 3-2. Wound-healing migration assay in the HCT116 cell line. The assay was performed in 6-well plates in triplicate 24 hrs after transfecting cells with EV and CD24^{+}. Photos were taken in each condition **(A)**; immediately after scratching (T=0) and 24 h later (T=24). The images were analysed using T-scratch software. The graph **(B)** shows a significant increase in cell motility with CD24+ compared to EV at T=24 ($p<0.05$). The data were analysed using Prism Pad 6 software (unpaired t-test).

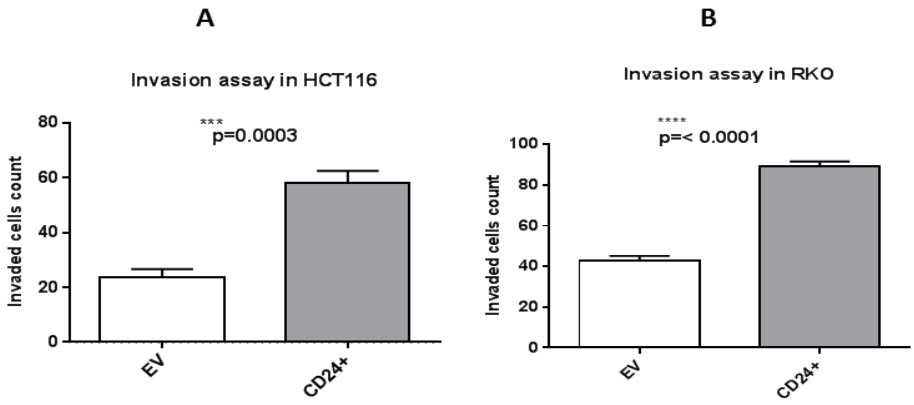


Figure 3-3. Matrigel invasion assay in CD24-negative CRC cell lines. Forced expression of CD24 performed in HCT116 and RKO cell lines and the assays carried out 24 hrs later. Compared to the EV control, overexpression of CD24 in both cell lines significantly induced cell invasion through Matrigel ($p<0.05$). The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

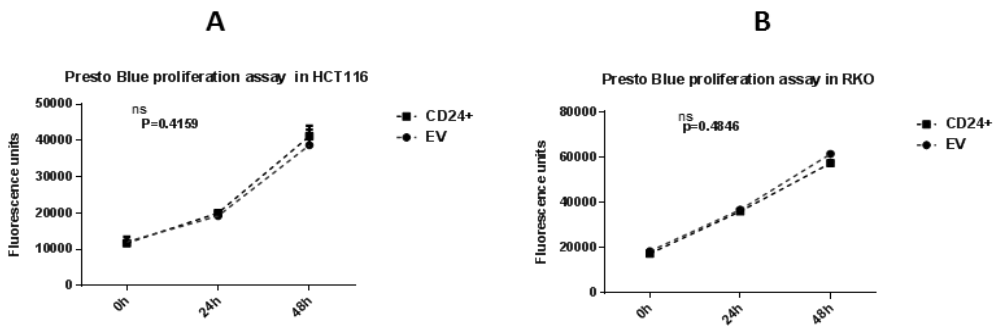


Figure 3-4. The effect of CD24 on cell proliferation in CD24-negative CRC cell lines. Forced expressed CD24 in HCT116 and RKO did not display a change in cell proliferation. The assay was performed in triplicate, and data were analysed using Prism Pad 6 software (two-way ANOVA).

The above data show that forced expression of CD24 in CD24-negative CRC cell lines (*i.e.* HCT116 and RKO) enhanced cell motility and invasion *in vitro*. To validate these results, we performed the complementary test of reducing levels of CD24 in cells with high endogenous expression of CD24 and measuring the effect of this on cellular functions, such as motility, invasion, and proliferation. The reduction in CD24 was achieved by transfecting CD24-specific siRNA into cells and comparing with cells transfected with siRNA which was specific for luciferase. Reduction of CD24 levels resulted in a significant reduction in Transwell cell motility in the SW620 and HT29 cell lines ($p=0.0002$; Figure 3-5A&B). Similarly, the Matrigel invasion assay in the above cell lines showed a significant reduction in cell invasion following CD24 siRNA transfection compared to luciferase siRNA (SW620 $p=0.0004$ and HT29 $p=0.0003$; Figure 3-6A&B). These data suggest that CD24 is able to stimulate both cell migration and invasion.

The PrestoBlue proliferation assay was also performed and this demonstrated that the knockdown of CD24 did not affect the cell proliferation rate in SW620 (p=0.20; Figure 3-7A), although, unexpectedly, it was significantly reduced following a similar transfection in the HT29 cell line (p=0.0004; Figure 3-7B).

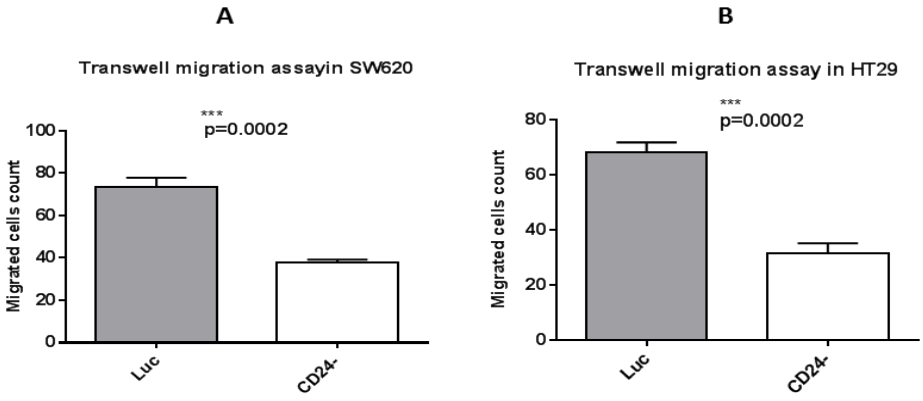


Figure 3-5. Transwell migration assay in CD24-positive CRC cell lines. The assays were performed 48 hrs after transfecting SW620 and HT29 cells with CD24-specific siRNA (CD24-) and luciferase siRNA (Luc). The data show that knockdown of CD24 in both cell lines resulted in a significant reduction in cell migration ($p<0.05$). The assay was performed in triplicate and the data were analysed using Prism Pad 6 software (unpaired t-test).

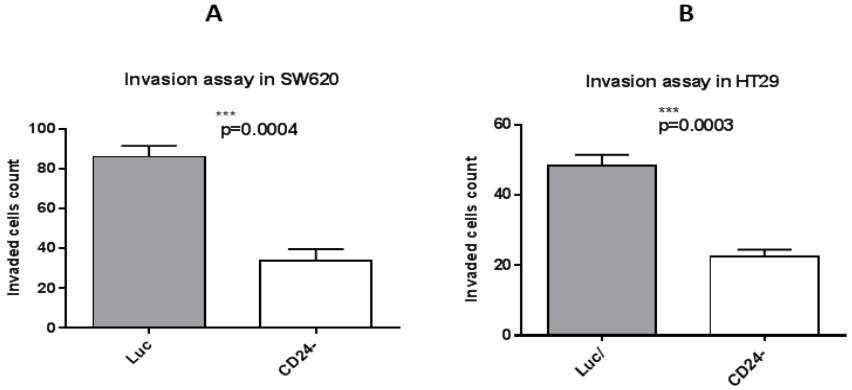


Figure 3-6. Matrigel invasion assay in CD24-positive CRC cell lines. SW620 and HT29 cells were transfected with CD24– and luciferase siRNA, and 48 hrs later, the assays were performed. The knocked-down CD24 significantly reduced cell invasion in both cell lines ($p<0.05$). The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

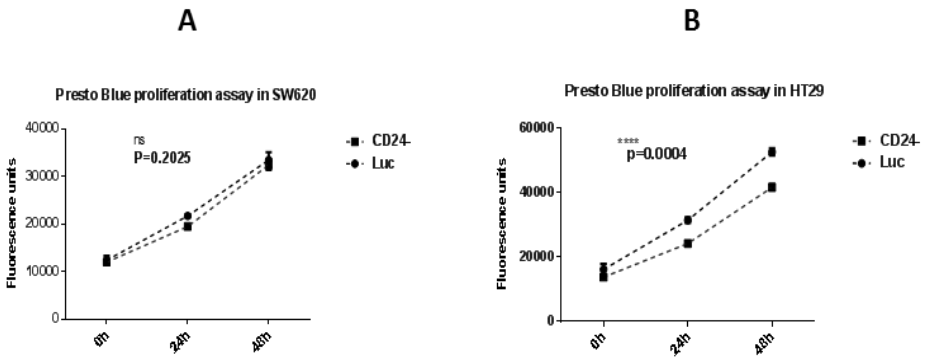


Figure 3-7. The effect of CD24 on cell proliferation in CD24-positive CRC cell lines. A significant reduction in cell proliferation was observed after knocking down CD24 in HT29 ($p<0.05$), but no effect was detected in SW620. The assay was carried out in triplicate, and the data were analysed using Prism Pad 6 software (two-way ANOVA).

Another feature required for metastasis is "stemness". The soft agar colony formation assay measures anchorage independent growth of tumour cells and is commonly used to indirectly evaluate stemness. This colony formation assay was performed in HCT116 and SW620 cells after manipulation of CD24 levels to detect whether CD24 can enhance anchorage-independent growth *in vitro*. The overexpression of CD24 in HCT116 significantly enhanced cluster formation ($p=0.0002$; Figure 3-8A&B). Similarly, the number of the colonies was significantly reduced following down-regulation of CD24 in SW620 ($p=0.0021$; Figure 3-8C&D). This finding indicates that CD24 is associated with colony formation in CRC, and therefore it may confer features of stemness.

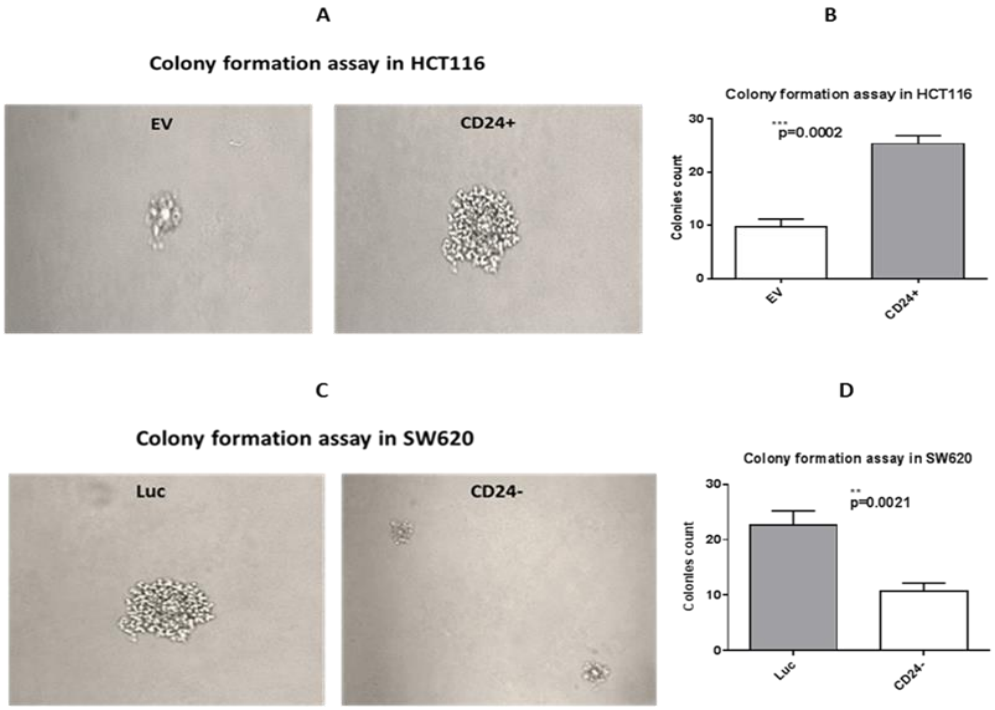


Figure 3-8. Soft agar colony formation assay in CRC cell lines. HCT116 and SW620 cells were transfected with either $CD24^{+}$ and EV plasmids or CD24–siRNA and luciferase-siRNA (Luc) for 48 hrs followed by the addition of cell suspension within the agarose layers in 6-well plates and incubation for 3 weeks at 37°C. Colony formation increased significantly following forced expression of CD24 in HCT116 ($p<0.05$), while it reduced significantly as a result of knockdown of CD24 in SW620 ($p<0.05$). The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

3.2.2 Investigation of the Role of CD24 in Regulating Cell Functions in Pancreatic Cancer

Since CD24 has been shown to play a role in regulating cell functions in CRC, it is imperative to further investigate this potential role in another cancer model. Pancreatic cancer usually arises from the pancreatic ducts and, in common with CRC, it is of endodermal origin and has frequent mutation of *TP53*, *SMAD4*, and *KRAS* (Hahn et al., 1996, Dergham et al., 1997). Thus, three pancreatic ductal cancer cell lines (Panc-1, PSN-1 and COLO357) were used and the expression levels of CD24 in their wild-type lysates were evaluated by Western

blot. CD24 protein was detected in PSN-1 and COLO357 but not in Panc-1. This allowed a similar dual approach as was used in the CRC cell lines i.e. forced expression CD24 in a Panc-1 and CD24 knockdown in PSN-1 and COLO357 to assess the potential effect of CD24 on cell function in pancreatic cancer.

Forced expression of CD24 in Panc-1 resulted in significant increases in both cell motility (using both Transwell migration and wound healing assays, p=0.007 and p=0.0079 respectively; Figures 3-9A & 3-10A&B) and cell invasion (p=0.0017; Figure 3-9B).

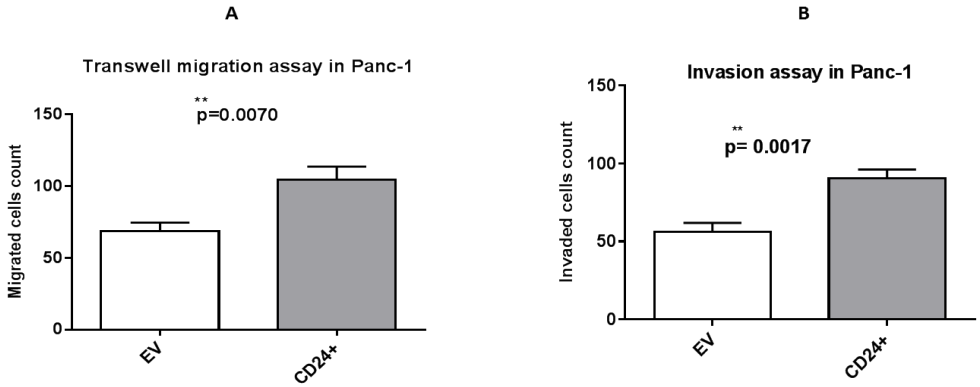


Figure 3-9. Transwell migration and Matrigel invasion assays in Panc-1. Both Transwell migration and Matrigel invasion assays were performed in Panc-1. CD24 overexpression in Panc-1 caused a significant increase in cell migration and invasion ($p<0.05$). The assay was repeated three times, and the data were analysed using Prism Pad 6 software (unpaired t-test).

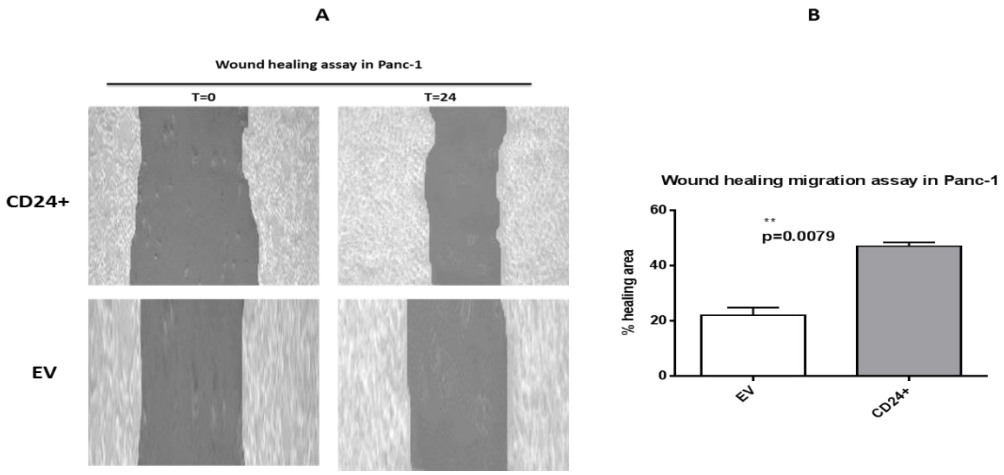


Figure 3-10. Wound-healing migration assay in the Panc-1 cell line. The images taken after scratching and (T=0) and 24 hrs later (T=24) were analysed using T-scratch software. The cell motility increased 24 hrs following transfection with $CD24^{+}$ compared to EV at T=0 ($p<0.05$). The data were analysed using Prism Pad 6 software (unpaired t-test).

In validation of the observations made following overexpression of CD24 in CD24-negative cell lines, CD24 down-regulation in PSN-1 and COLO357 caused a significant reduction in cell motility (p=0.0002 and p=0.0003, respectively; in the Transwell migration assay; Figure 3-11A&B) and invasion (p=0.0004 and p=0.0006, respectively; Figure 3-12A&B).

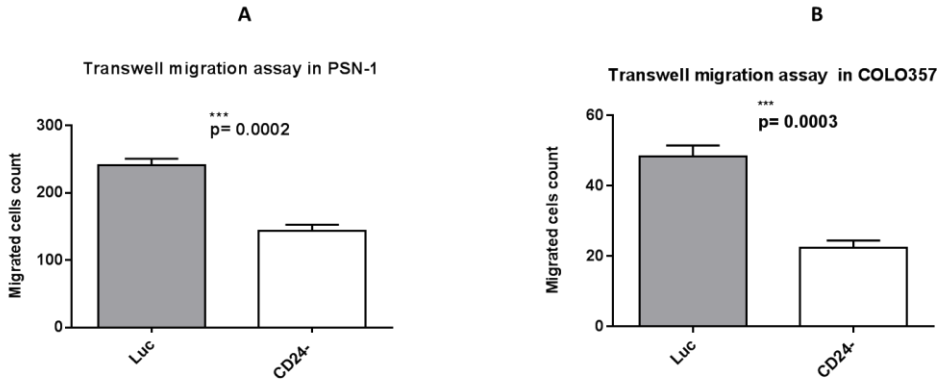


Figure 3-11. Transwell migration assay in PSN-1 and COLO357. A significant reduction in cell motility ($p<0.05$) was detected following the down-regulation of CD24 in these cell lines. The assay performed in triplicate data were analysed using Prism Pad 6 software (unpaired t-test).

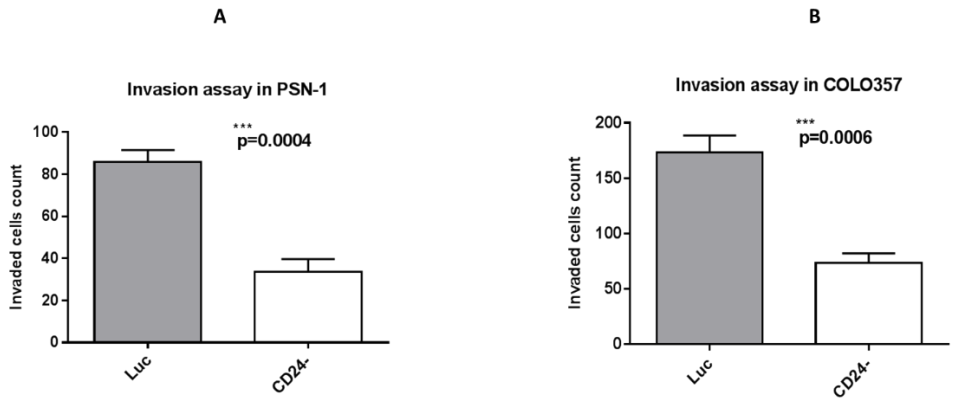


Figure 3-12. Matrigel invasion assay in PSN-1 and COLO357. CD24 knockdown in these cell lines resulted in a significant reduction in cell invasion ($p<0.05$). The assay performed in triplicate data were analysed using Prism Pad 6 software (unpaired t-test).

In contrast with the some observations in CRC, changes in CD24 expression consistently altered cell number. Forced expression of CD24 in Panc-1 cells resulted in a significant increase in cell proliferation rate ($p=0.0082$, Figure 3-13A), while knockdown of CD24 in PSN-1 and COLO357 cells resulted in a significant reduction in cell proliferation ($p=0.0005$ and $p=0.0003$, respectively; Figure 3-13B&C).

The soft agar colony formation assay performed in Panc-1 and PSN-1 showed that colony formation is consistently influenced by CD24 levels. It was observed to be significantly enhanced as a result of forced expression of CD24 in Panc-1 ($p=0.0018$; Figure 3-14A&B), while it was significantly reduced following knockdown of CD24 in PSN-1 ($p=0.0003$; Figure 3-14C&D).

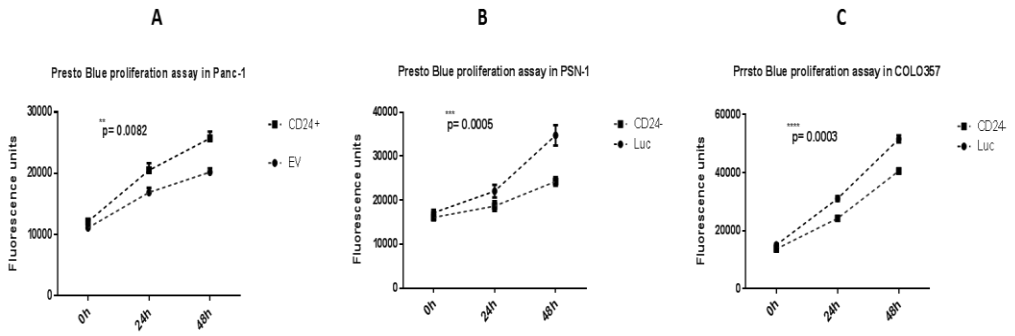


Figure 3-13. The effect of CD24 on cell proliferation in pancreatic cancer. PrestoBlue proliferation assay was done individually in three pancreatic cell lines, Panc-1, PSN-1 and COLO357. In a comparison to the control, forced expression of CD24 in Panc-1 caused a significant increase in cell proliferation ($p<0.05$, A). CD24 knockdown in PSN-1 and COLO357 significantly decreased cell proliferation levels ($p<0.05$, B&C). The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (two-way ANOVA).

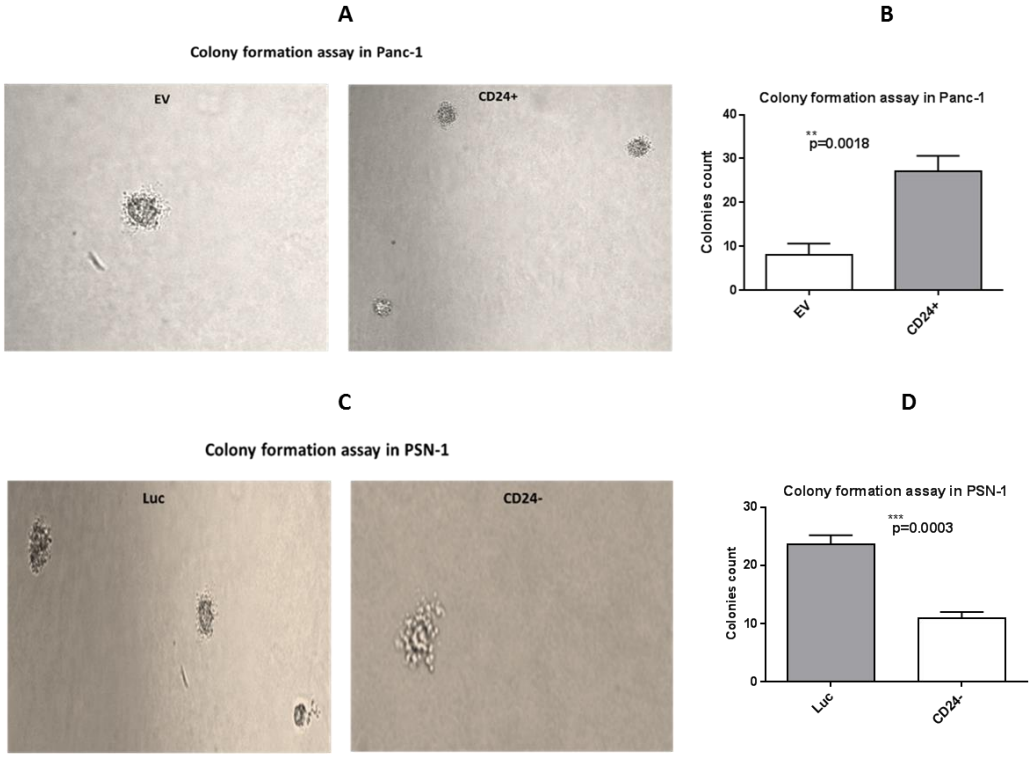


Figure 3-14. The soft agar colony formation assay in pancreatic cancer cell lines. The colony formation level was significantly increased following transfection of Panc-1 cells with the CD24 plasmid in comparison to the EV control ($p<0.05$), while it was significantly reduced following knockdown of CD24 in PSN-1 ($p<0.05$). The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

3.2.3 Investigation of the Role of CD24 in Regulating cell Functions in Hepatocellular Cancer (HCC)

HCC and cholangiocarcinoma are cancers of the liver. HCC arises from the hepatocytes while cholangiocarcinoma arises from the biliary epithelium. Hepatocytes arise from the embryonic endoderm and, in common with CRC, the HCCs show activation of *Wnt* signalling and frequent mutation of *TP53, KRAS,* and *SMAD4* (Hahn et al., 1996, Dergham et al., 1997). The hypothesis that CD24 expression may modulate metastasis in HCC was therefore tested in the HCC cancer cell line (HUH7). Initially, the protein expression level of CD24 was evaluated and it was found to be a CD24-positive cell line (see Appendices, Fig. 9-3). Thus, knockdown of CD24 in HUH7 cells and relevant functional studies were carried out. The generated data showed that down-regulated CD24 in HUH7 caused significant reductions in both cell motility (p=0.0001; Figure 3-15A) and invasion (p=0.0006; Figure 3-15B). However, the wound-healing migration assay performed following the transfection of HUH7 cells with CD24–siRNA did not show any effect on cell motility (p=0.069; Figure 3-16A&B).

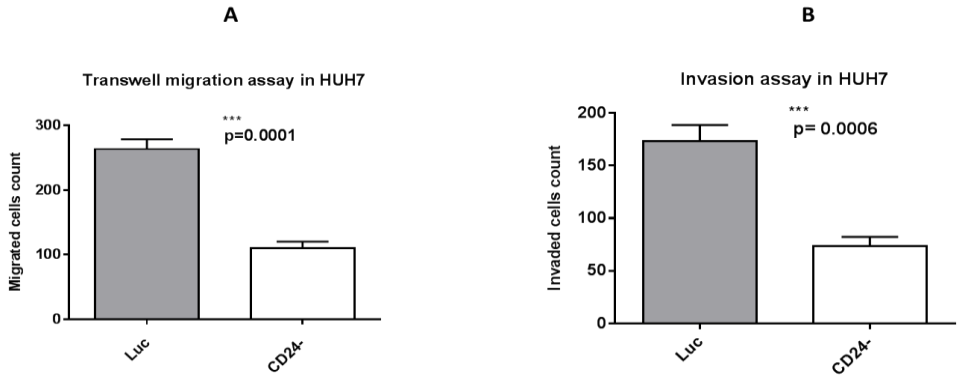


Figure 3-15. Transwell migration and Matrigel invasion assays in HUH7. CD24 knockdown in HUH7 resulted in a significant reduction in cell motility and invasion ($p<0.05$). The assay was performed in triplicate, and data were analysed using Prism Pad 6 software (unpaired t-test).

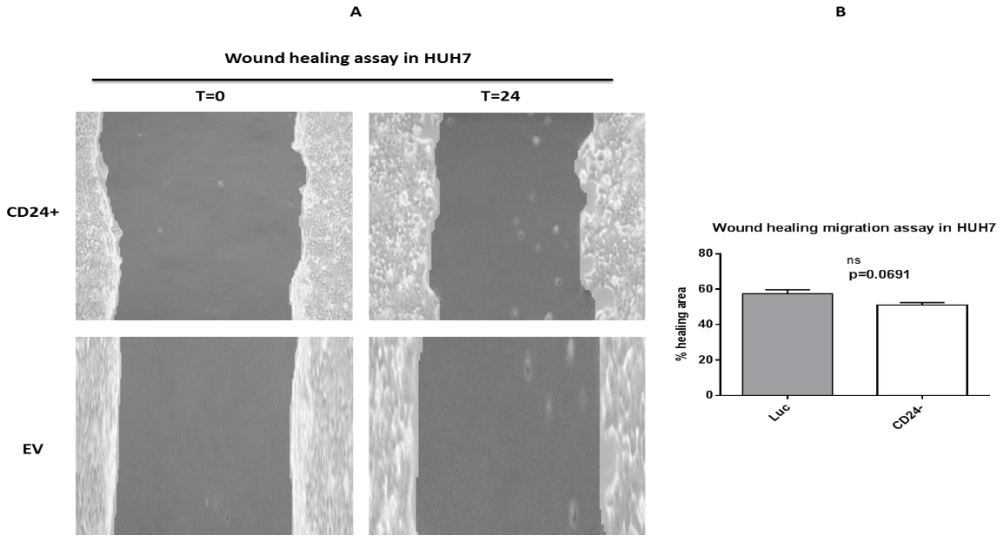


Figure 3-16. Wound-healing migration assay in the HUH7 cell line. The images (T=0 and T=24) were analysed using T-scratch software. No significant percentage in wound healing was observed 24 hrs following knockdown of CD24 ($p \geq 0.05$). The data were analysed using Prism Pad 6 software (unpaired t-test).

PrestoBlue proliferation assay data showed that CD24 regulates cell proliferation in HCC, as the knockdown of CD24 in HUH7 resulted in a significant reduction in cell proliferation (p=0.0022; Figure 3-17). The number of colonies formed on the soft agar decreased significantly following the down-regulation of CD24 in HUH7 (p=0.0009; Figure 3-18A&B). These findings suggest that CD24 modulates cell functions in HCC.

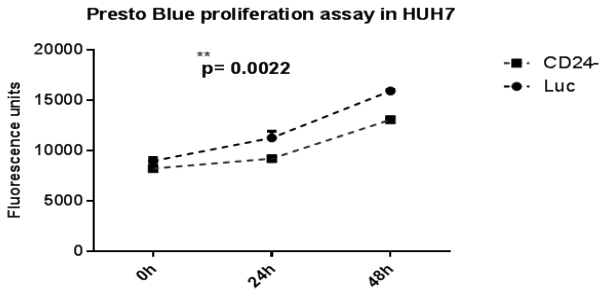


Figure 3-17. The effect of CD24 on cell proliferation in HCC. PrestoBlue proliferation assay was performed in HUH7 cell line. In a comparison to the control, CD24 knockdown in HUH7 led to a significant decrease ($p<0.05$) in the cell proliferation level. The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (two-way ANOVA).

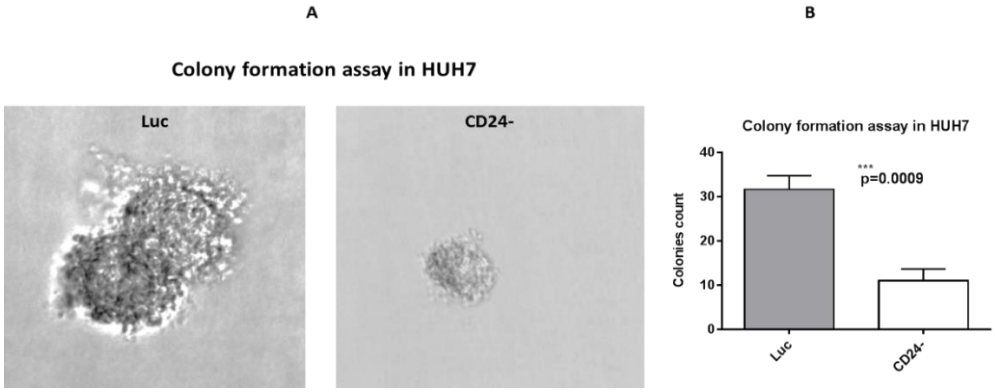


Figure 3-18. Soft agar colony formation assay in HCC. The number of colonies formed on the soft gel decreased significantly after knockdown of CD24 in HUH7 compared to the control ($p<0.05$). The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

3.2.4 Investigation of the Role of CD24 in Regulating Cell Functions in Lung Cancer

Our consistent observations in CRC, pancreatic and liver cancer lend support to the notion that CD24 may regulate cell functions in lung cancer. Lung tissue is also derived from embryonic endoderm and non-small cell lung cancer (NSCLC) have, in common with CRC, frequent driver mutations affecting *EGFR* / *KRAS* / *BRAF* / *TP53* (Cai et al., 2014). Given these facts, we hypothesised that CD24 regulates cell behaviour in lung cancer via the same mechanism described in the three-cancer model delineated above.

To test this hypothesis, three NSCLC cell lines (A549, H460 and H226) were identified for *in vitro* functional studies, and CD24 expression levels in their wild-type lysates were evaluated using a Western blot test. CD24 protein was detected in A549 and H226 but not in H460 (see Appendices, Fig. 9-3); thus, CD24 was overexpressed in H460 and knocked down in the other cell lines for further experimental work. Every experiment was repeated three times to assess the likely effect of CD24 on cell function in lung cancer.

Consistent with the observations in the other cancer models, the functional studies showed that overexpression of CD24 in H460 led to significant increases in Transwell migration and wound healing cell motility (p=0.0006 and p=0.019; Figures 3-19A & 3-20, respectively) and cell invasion (p=0.0001; Figure 3-19B). Knockdown of CD24 in H226 and A549 resulted in significant reductions in Transwell cell motility (p=0.0005 and p=0.0024, respectively; Figure 3-17C&E) and cell invasion (p=0.0001and p=0.0007, respectively; Figure 3-19D&F).

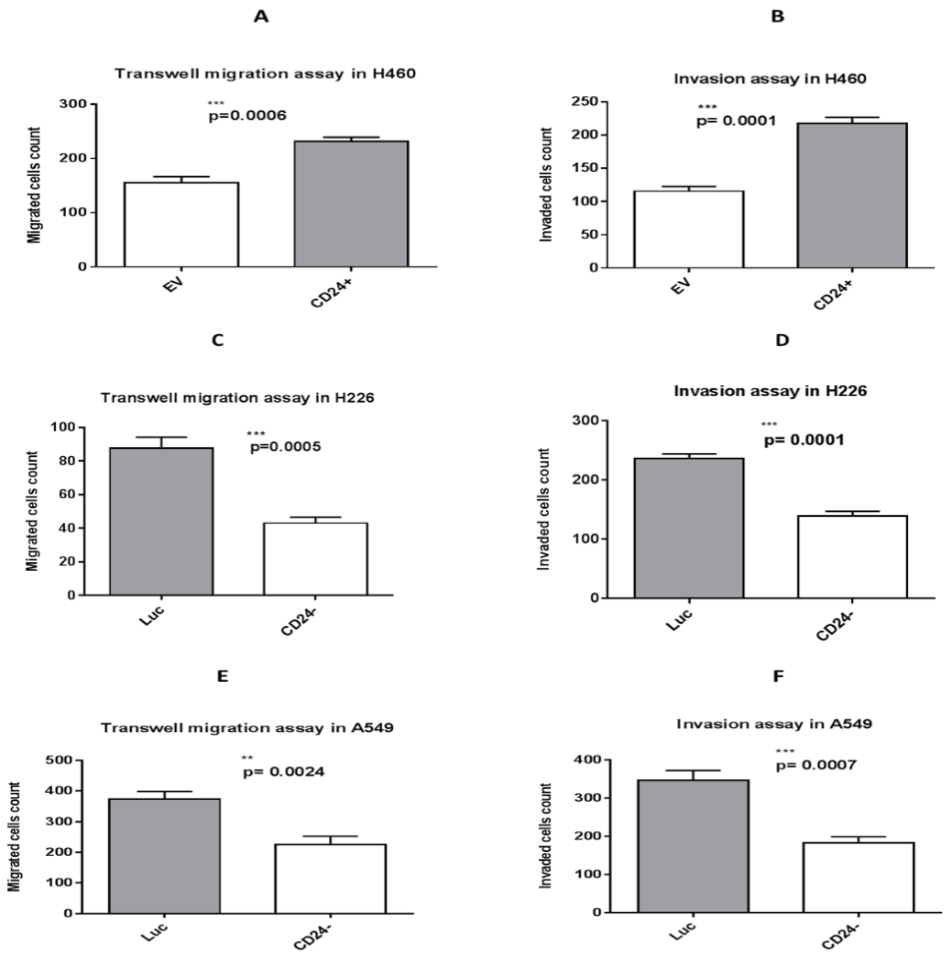


Figure 3-19. Transwell migration and Matrigel invasion assays in NSCLCs. The overexpression of CD24 in H460 significantly enhanced cell motility and invasion ($p<0.05$; A&B). The knockdown of CD24 in H226 and A549 caused a significant increase in cell migration ($p<0.05$, Fig. C&E) and invasion ($p<0.05$, D&F). The assays were performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

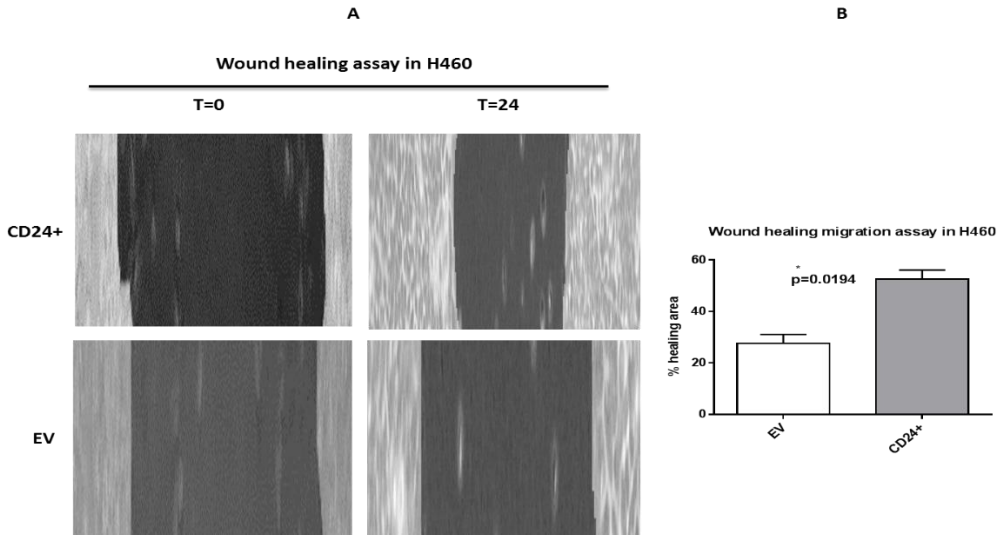


Figure 3-20. Wound-healing migration assay in the H460 cell line. The images were analysed using T-scratch software. A significant increase in cell motility with $CD24^+$ compared to EV at T=24 ($p<0.05$). The data were analysed using Prism Pad 6 software (unpaired t-test).

Overexpression of CD24 in H460 caused a significant rise in the cell proliferation rate (p=0.027; Figure 3-21A), while knockdown of CD24 in A549 caused a significant reduction in cell proliferation (p=0.0004; Figure 3-21B). The soft agar colony formation assay carried out in H2460 and A549 illustrated that the colonies count has affected significantly in proportional with the activity of CD24 (p=0.0005 and p=0.0008, respectively; Figure 3-22A–D). Together, these findings are consistent with our results in the above three-cancer model and suggest that CD24 modulates cell functions in lung cancer.

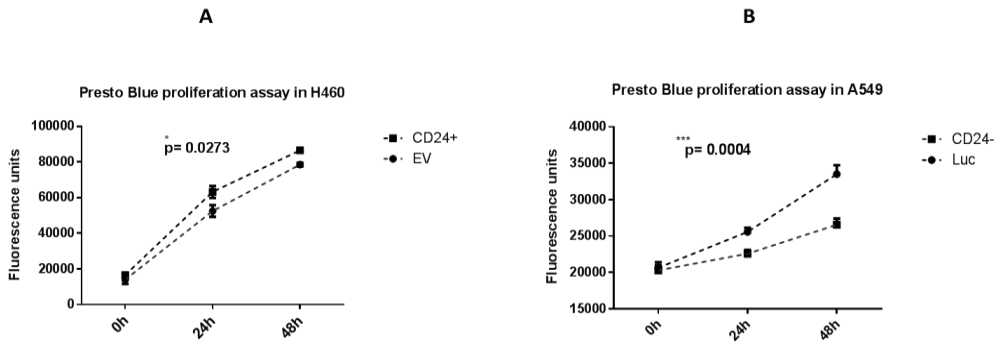


Figure 3-21. The effect of CD24 on cell proliferation in lung cancer. Forced expression of CD24 in H460 resulted in a significant increase in cell proliferation level ($p<0.05$). Knockdown of CD24 in A549 led to a significant decrease in cell proliferation ($p<0.05$). The assay was performed in triplicate, and data were analysed using Prism Pad 6 software (two-way ANOVA).

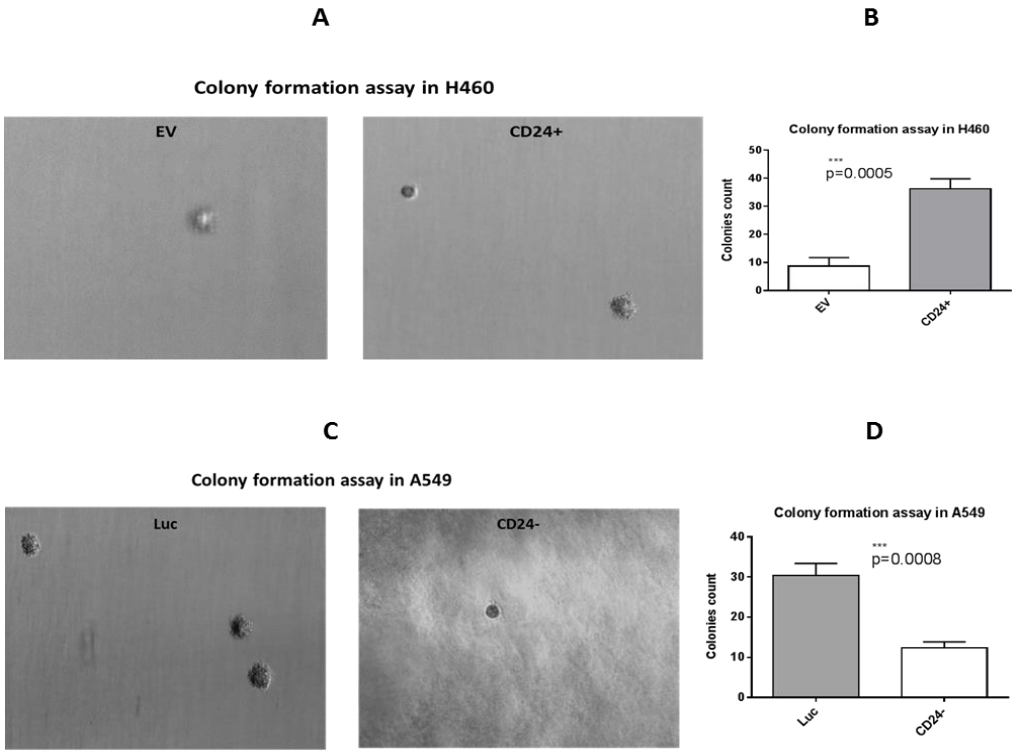


Figure 3-22. The soft agar colony formation assay in lung cancer. The number of colonies on the soft gel increased significantly following overexpression of CD24 in H460, while they decreased following knockdown of CD24 in A549 compared to the control ($p<0.05$). The assay was performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

3.3 Discussion

Due to its attachment to the cell surface via the GPI anchor, localisation in the lipid raft domain and binding to extracellular matrix components, CD24 can regulate many cellular functions (Baumann et al., 2005). These properties are highly relevant in terms of cancer progression and metastasis and thus suggest that CD24 may be an important factor in these processes.

The biological function of the CD24 gene in the development of cancer was studied. The primary interest of our research group is the study of colorectal cancer. However, in order to broaden the scope of the research, the function of the CD24 gene in other cancer models was studied further. Pancreatic, HCC and lung cancer were chosen, as common factors exist in the properties of these cancers and those of colorectal cancer (Sagiv et al., 2008, Hahn et al., 1996, Mahlamaki et al., 2002).

Throughout this chapter, the potential effect of CD24 on cell function in the four cancer models has been investigated. The levels of CD24 protein were altered and the effect of this change was evaluated using several cell function assays. All techniques have limitations and therefore, in order to ensure that the data were robust, CD24 was altered by forced expression and by gene knockdown. Before proceeding to study the effect of manipulated CD24 in these cancer models, appropriate cell lines for each cancer model had to be selected. For CRC, pancreatic cancer and lung cancer, cell lines with low CD24 expression (suitable for forced expression study) and high CD24 expression (suitable for gene knockdown study) were identified. For HCC, only a high CD24 expressor cell line was identified.

CD24 Stimulates Cell Migration and Invasion

Cell motility was evaluated using assays for cell migration (Transwell migration and wound healing) and for cell invasion (migration through Matrigel). Previous data from our group have shown that high levels of CD24 are associated with increased cell motility (Ahmed et al., 2010). These results were validated and it was shown that, in the cell lines HCT116 and RKO, increasing the levels of CD24 resulted in an increase in both cell migration and cell invasion. In contrast, reducing CD24 levels the cell lines SW620 and HT29 resulted in a reduction of cell motility.

These data were confirmed in the other cancer models tested and thus provided a very strong case for the role of CD24 in stimulating both migration and invasion. Some variation in the effect of CD24 on cell proliferation was noted and it is possible that this may have affected the data on cell motility. In CRC, however, no effect on cell proliferation in three cell lines was observed and therefore these data can be taken at face value. From this, it can be inferred that the effects observed in the other cancer models are true effects. However, to be absolutely certain, these studies would need to be repeated with a mitosis inhibitor such as paclitaxel or colchicine.

A large body of literature has reported that CD24 can alter cell functions, including cell motility and invasion, in various solid cancers (Baumann et al., 2005, Jaggupilli and Elkord, 2012). However, the mechanism through which CD24 can stimulate cell motility is uncertain and is partially investigated in the next chapter. Previous studies showed that CD24, in common with other GPI-linked proteins, also correlates with signal transduction mechanisms and interacts with Src family members in different tumour cell lines (Zarn et al.,

1996, Sammar et al., 1997). A recent study reported that both cell migration and invasion were affected due to the inhibition of Src expression in CRC tissues (Muppala et al., 2013). Consistent with the above observations, Src was found to associate with CD24 expression in different cancer models (as shown in the next chapter), suggesting that it might be a downstream target through which CD24 regulates cell migration and invasion.

In breast cancer cells, inducing CD24 expression enhanced cell migration and invasive properties, although enhanced migration was not found to be fully dependent on the activity of α3β1 or α4β1 integrins. These results suggest that CD24 stimulates migration in two ways: first, by promoting adhesion to a substrate in an integrin-dependent manner, and second, by stimulating cell movement in an integrin-independent manner. Direct interaction between CD24 and β_1-containing integrins has been reported, indicating that CD24 may stimulate integrin activity and therefore induce cell behaviour, including cell migration and invasion (Baumann et al., 2005). In addition, FAK activation has been reported to play a crucial role in tumour development and metastasis (Schwartz and Assoian, 2001). Therefore, FAK could be a significant mediator of CD24-dependent cellular behaviour.

Cytoskeleton remodeling is also essential for the regulation of cell migration and invasion (Hall, 2009). Overexpression of CD24 was found to induce actin cytoskeletal remodeling activity and to increase contractile forces to facilitate cell invasion (Zhou et al., 2013). An association between CD24 and CXCR4 in cholangiocarcinoma was also found. This observation may indicate that the CXCR4-mediated activation of signalling pathways causes increased cancer cell

migration and invasion, which correlated with actin polymerization and matrix metalloproteinase-9 (MMP-9) activation (Leelawat et al., 2013).

CD24 Stimulates Cell Proliferation

Cell proliferation was measured over 48 hrs using the PrestoBlue proliferation assay, which provides better data, since the cells are followed for the whole assay compared to methylene blue. Previous data from our group have shown that CD24 does not affect cell proliferation after manipulating CD24 expression in some CRC cell lines (Ahmed et al., 2010). In this study, CD24 seems not to affect cell proliferation in 75% of CRC cell lines were used; therefore it is probably not significant in CRC. In contrast, it is important in the other cancer models, since modulation of CD24 expression resulted in significant changes in cell proliferation. Despite substantial evidence that CD24 is involved in the regulation of cell proliferation, the mechanisms by which it does so are still unclear.

CD24 was reported to be targeted by Ral GTPases, which suggests that CD24 may mediate cell proliferation (Smith et al., 2006). Another study (Bretz et al., 2012b) reported that the activity of Src kinase is stimulated by CD24, and that Src is involved in activation of STAT3. Once activated, STAT3 is dimerised and translocated into the nucleus. The DNA binding of the STAT3 dimers initiates the expression of specific target genes that may alter cell features, including cell proliferation. A further study suggested that the link between CD24 and MAPK pathways might disclose a novel mechanism in the regulation of cell proliferation in CRC (Wang et al., 2010). In breast cancer, CD24 was found to regulate cell proliferation via modulation of integrin β1 stability (Lee et al., 2012).

CD24 Stimulates Colony Formation

CD24 has been shown to stimulate the anchorage-independent growth of tumour cells "stemness" (Bretz et al., 2012b). The potential association between CD24 expression and colony formation in the four cancer models was evaluated *in vitro* using the soft agar colony formation assay. Results showed that CD24 is associated with colony formation in these cancer models, and thus may confer features of stemness. However, the mechanism by which CD24 might stimulate the anchorage-independent growth of tumour cells needs to be identified. A previous study suggested that CD24 could regulate stemness by signalling through the modulation of Notch1 mRNA stability by p38MAPK (Lim et al., 2014). In HCC, stemness was found to be promoted by miR-205, which targets phospholipase C Beta 1 (PLCβ1) and enhances CD24 expression. Furthermore, it has been shown that CD24 activates STAT3 and Src activity, enhancing cancer-promoting activities involved in stemness and metastasis (Bretz et al., 2012b).

In summary, the data presented here show for the first time that CD24 consistently regulates cell functions in various tumour cell lines, possibly via a unique mechanism. Such consistency between the observations in the four cancer models could have a number of important implications for future practice and may help to identify the mechanism(s) by which CD24 regulates cell functions. Overall, CD24 seems to have all the features necessary to promote metastasis. Further functional studies need to be carried out to validate the significance of CD24 in regulating other cell functions, such as cell adhesion, differentiation, and apoptosis.

4 INVESTIGATING THE DOWNSTREAM TARGETS OF CD24 IN CANCER

4.1 Introduction

In chapter 3, our data suggested that the CD24 protein plays an active role in regulating various cancer cell functions including cell motility, invasion and proliferation (Rostoker et al., 2015). In addition, a substantial proportion of the literature reports that the CD24 gene has a role as a putative cancer stem cell marker, a novel oncogene, and a prognostic factor for poor outcomes in several cancers (Liu et al., 2011, Yang et al., 2014). These significant features stimulate cancer cells to spread further and form malignant colonies which are distant from the original site of infection (Nguyen, 2011). Nonetheless, it is still unclear how these vital cellular processes are regulated by CD24, and by which mechanism(s) this molecule may be controlled in cancer.

In attempts to further identify downstream targets of CD24, we thought that the effects of CD24 on cell motility may be dependent on its association with integrins. Since CD24 is a GPI anchored protein, it does not have an intracellular domain and therefore must rely on other molecules for transmitting external signals into the cell. The localisation of CD24 to lipid rafts in association with integrins raises the possibility that this may be a mechanism for CD24 signalling and activity. The proteins focal adhesion kinase (FAK) and integrin-linked kinase (ILK) are well-studied effectors of integrin activity and it appears that ILK associates with and is influenced by diverse signalling pathways (Dedhar et al., 1999). Our lab has previously shown that ILK and FAK are downstream targets of Cten (also known as Tensin 4). Cten is also associated with integrins and unpublished data from our lab using a phosphokinome array have suggested that CD24 and Cten may have a number of common targets.

We hypothesised that CD24 may mediate part of its activity through signalling pathways centred around integrins and integrin associated molecules such as Cten. To investigate the above hypothesis, CD24 was manipulated in the four-cancer (colorectal, pancreatic, liver, and lung cancer) model cell lines. Changes in the expression of molecules identified as regulating cell functions such as cell migration, invasion, and anchorage independent cell growth were evaluated. Western blotting and qPCR techniques were used to test molecules such as Cten, FAK, ILK, AKT, and EMT markers including N-cadherin, E-cadherin and Snail, in addition to other possible associated molecules. Additionally, different applicable assays were performed to confirm possible interaction between CD24 and Cten to determine if these proteins could physically interact with each other in a complex protein.

Formation of metastasis requires movement of tumour cells to a new site and growth of tumour cells at the new site. This may require features of stemness and our data had shown that CD24 stimulates colony formation – a commonly used marker for stemness. We thus investigated the mechanism through which CD24 may confer features of stemness.

4.2 Results

4.2.1 Investigation of CD24 Downstream Targets in CRC

4.2.1.1 CD24 Forced Expression in Negative/Low Expressor CRC Cell Lines

CD24 was forcibly expressed with pcDNA3.1-CD24 plasmid in the CRC cell line (HCT116), which is a low expressor of CD24, since the Western blot test confirmed that HCT116 slightly expresses CD24 and Cten. The Western blot results (Figure 4-1, A) showed that overexpressed CD24 in the above cell line

resulted in a notable increase in the expression levels of Cten and also in ILK and FAK (i.e., molecules that were previously identified as downstream targets of Cten). To validate these observations and to further confirm the association between CD24 and Cten, ILK, and FAK in CRC, CD24 was overexpressed in another CD24/Cten-negative cell line (RKO). We noticed that the expression level of Cten, FAK, and ILK had increased after overexpression of CD24 (Figure 4-2, B). These findings are similar to those observed in HCT116, suggesting that Cten, FAK, and ILK are downstream targets of CD24.

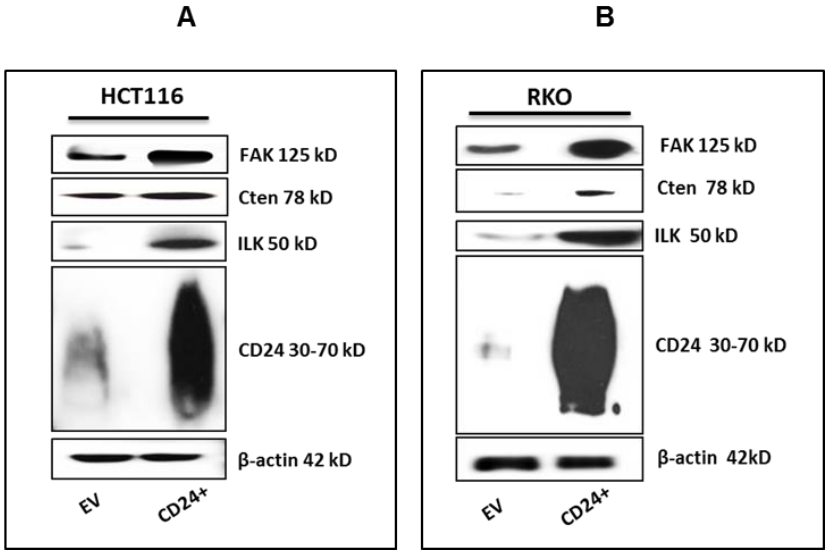


Figure 4-1. Western blot of CD24 following forced expression in CRC cell lines. CD24 forced expression was performed in CD24 low/negative cell lines, HCT116 **(A)** and RKO **(B)**, using $CD24^{+}$ and EV plasmids to evaluate the expression level of potential downstream targets. Western blot shows that this led to an increase in the expression level of Cten as well as in FAK and ILK expression. The loading control protein (β-actin) showed the amount of proteins loaded on the gel (25 μg). All the experiments were done in triplicate.

4.2.1.2 CD24 Knockdown in Moderate/High Expressor CRC Cell Lines

The above findings illustrated that CD24 forced expression in both HCT116 and RKO cell lines has influenced the expression levels of Cten, FAK, and ILK. Therefore, the next step was to validate these results by knocking down CD24 in CD24 moderate/high expressor CRC cell lines. CD24 was knocked down

using siRNA in CRC cell lines (SW620 and HT29), which are high and moderate expressors of CD24, respectively. The data generated by the Western blot test after each transfection in these cell lines demonstrated that down-regulation of CD24 resulted in reduction of the expression levels of Cten along with ILK and FAK (Figure 4-2, A & B). These findings confirm the association between CD24 expression and these potential downstream targets.

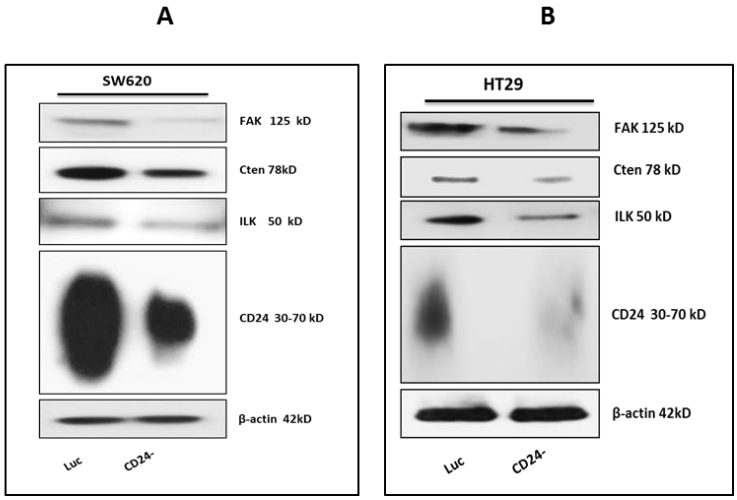


Figure 4-2. Western blot of CD24 knockdown in CRC cell lines. CD24 knocked down in the CD24 high and moderate expressors CRC cell lines: SW620 **(A)** and HT29 **(B),** using siRNA and targeted to CD24 and, as a control, luciferase siRNA. This caused a reduction in the expression level of Cten as well as in the expression of FAK and ILK in both cell lines. The loading control protein (β-actin) showed the amount of proteins loaded on the gel (25 µg). Transfections and Western blot experiments were repeated three times.

4.2.1.3 The Relationship between CD24 and Cten

Our data showed that changes in the levels of CD24 were followed by changes in the levels of Cten, ILK and FAK. It is possible that each of these is directly and independently positively regulated by CD24, but data from the lab have previously shown that both ILK and FAK are up-regulated by Cten (Al-Ghamdi, 2013). Since both CD24 and Cten are associated with integrins, we thought that it was most likely that CD24 was up-regulating Cten and this then had

downstream effects (such as the changes in ILK and FAK). In order to investigate whether CD24 was signalling through Cten, we forcibly expressed CD24 in HCT116 cell line and simultaneously knocked down Cten. We found that overexpression of CD24 led to a significant increase in the expression level of Cten. Unexpectedly the expression level of CD24 reduced following down-regulation of Cten although these cells had also been transfected with the CD24 plasmid construct (Figure 4-3, A).

Given the apparent effect of Cten knockdown on CD24 levels and the association of both molecules with integrins, it was possible that there could be mutual influence. To investigate this, we forcibly expressed Cten and simultaneously knocked down CD24. The Western blot data (Figure 4-3, B) showed that overexpression of Cten resulted in an augmentation in the expression level of CD24, while a reduction in the expression of Cten was noticed following knockdown of CD24, suggesting that CD24 may regulate and interact with Cten in CRC.

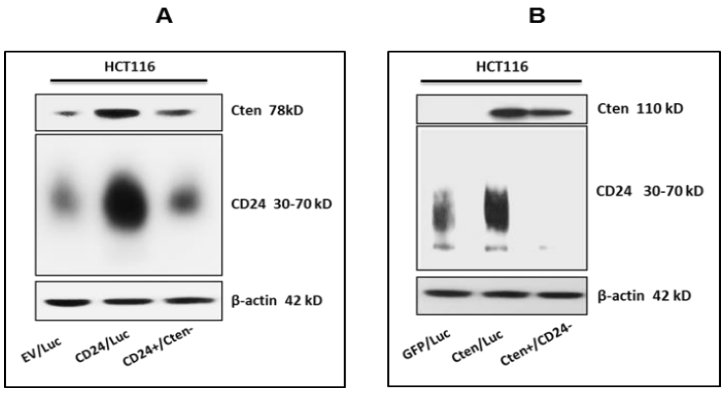


Figure 4-3. Western blots of co-transfection of CD24 and Cten in CRC cell lines. The expression level of Cten has significantly increased following overexpression of CD24 in HCT116 cell line, while a decrease in the expression level of CD24 was also observed after Cten knockdown **(A).** Overexpression of Cten led to an increase in the expression level of CD24, and the expression of Cten decreased following down-regulation of CD24 **(B).**

Given the apparent effect and unexpected effect of Cten on CD24 levels, it was necessary to check this in another cell line. Therefore, we knocked down Cten in the SW620 cell line (a high expressor for CD24 and Cten), and any change in the expression level of CD24 was then evaluated by Western blot. The data demonstrated that the Cten knockdown did affect CD24 expression (Figure 4-4), further supporting the idea that Cten could have a feedback impact on CD24 expression.

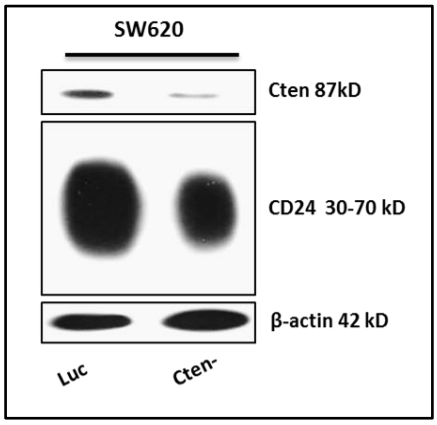


Figure 4-4. Western blot of Cten knockdown in CRC cell line. Cten knocked down in the SW620 cell line (a positive cell line for both Cten and CD24) using a specific siRNA. This procedure was performed to detect whether Cten might influence CD24 expression. A reduction in the expression of CD24 following Cten knockdown was noticed. The loading control (β-actin) showed the amount of proteins loaded on the gel (25 µg).

4.2.1.4 The Effect of Co-transfection of CD24 and Cten on Cell Functions

Although CD24 and Cten appeared to have a mutually stabilizing effect, it was necessary to ascertain whether the interaction was functionally relevant and, in particular, whether Cten was necessary for CD24 functional activity. Functional studies were carried out following co-transfection of one molecule (i.e., CD24 or Cten) and knockdown of the other (i.e., Cten or CD24) in the HCT116 cell

line. Transwell migration and Matrigel invasion assays were performed to detect whether CD24 and Cten might interact and hence regulate cell functions, and to assess if Cten (identified as a target of CD24) may have feedback loops on CD24 activity and consequently control cell activities. The data (Figure 4-5, A & B) showed that co-transfected $CD24^{+}$/Luc resulted in a significant increase in cell motility and invasion ($p < 0.0001$) compared with the control (EV/Luc). However, knockdown of Cten with CD24 forced expression (CD24+/Cten-) significantly reduced cell motility and invasion ($p = 0.0003$ and $p < 0.0001$, respectively) compared to the $CD24^{+}$/Luc.

Functional assays were performed following co-transfection of Cten and knockdown of CD24 in HCT116. Transwell migration assay results (Figure 5-4, C & D) demonstrated that co-transfected $Cten^{+}$/Luc in HCT116 caused a significant increase in cell motility and invasion ($p = 0.0001$ and $p < 0.0001$, respectively) compared with the control (GFP/Luc). However, co-transfected Cten and knockdown of CD24 (Cten+/CD24-) led to a significant reduction in cell motility and invasion ($p = 0.0002$ and $p < 0.0001$, respectively) compared to the co-transfected Cten ($Cten^{+}$/Luc). Together, these data suggest that Cten is targeted by CD24 signalling to promote cell motility in CRC. Cten may also have a feedback loop on CD24 activity and therefore regulate cell functions. However, it was observed that Cten activity is not entirely dependent on CD24.

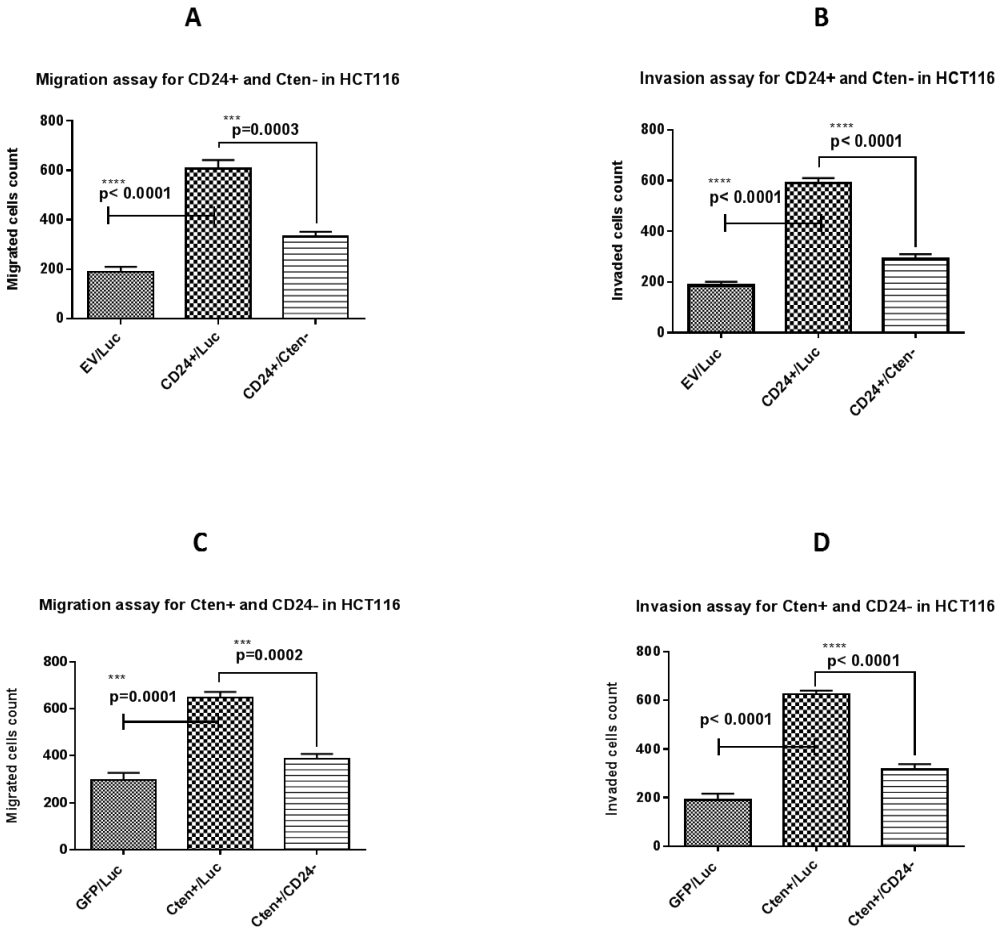


Figure 4-5. Functional assays of co-transfection of CD24 and Cten in CRC cell lines. Transwell migration **(A)** and Matrigel invasion **(B)** assays were performed after CD24 co-transfection with luciferase, and knockdown of Cten with siRNA in HCT116 cell line. CD24 overexpression caused a significant increase in cell migration and invasion rates ($p < 0.05$) compared to the control (EV/Luc). Likewise, knockdown of Cten resulted in a significant reduction in cell migration and invasion ($p = 0.0003$ and $p < 0.0001$, respectively), despite the presence of CD24. The same assays were carried out following Cten co-transfection and knockdown of CD24 in the same cell line **(C & D).** A significant increase in cell motility and invasion was found ($p < 0.05$) after overexpression of Cten compared with the control (GFP/Luc), while they reduced significantly ($p = 0.0002$ and $p < 0.0001$, respectively) after knockdown of CD24.

4.2.1.5 Investigation of the Regulation of Cten Gene Expression by CD24

Our data showed that Cten is regulated by CD24 at protein level. In order to find whether this was due to increase expression of the Cten gene, the expression of Cten at mRNA was evaluated following CD24 manipulation in CRC cell lines. CD24 was overexpressed in HCT116, while it was knocked down in SW620. qPCR for Cten mRNA levels in RNA samples extracted after each transfection did not demonstrate significant changes (p =0.154 and p=0.105, respectively) compared to the negative controls (Figure 4-6, A & B). This finding suggests that CD24 regulates Cten at the post-transcriptional level.

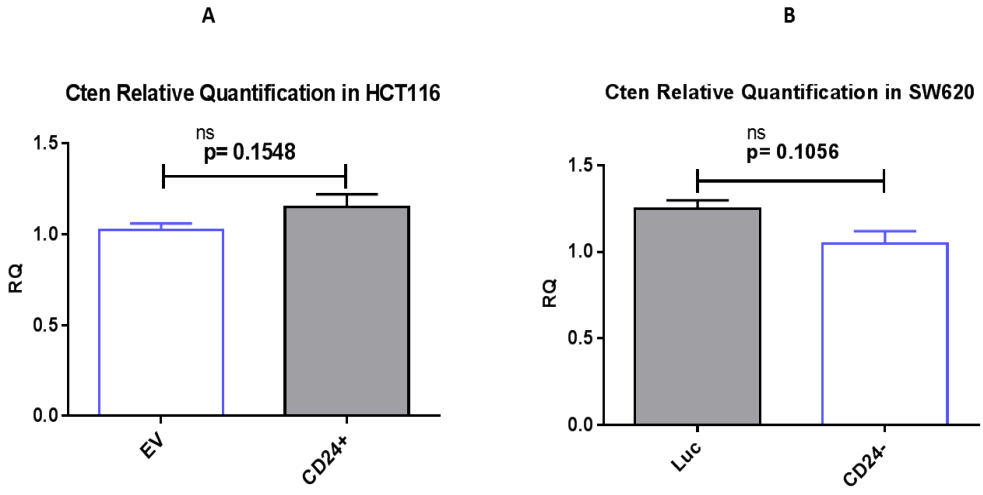


Figure 4-6. Cten relative quantification after CD24 manipulation in CRC cell lines. qPCR for Cten mRNA levels in RNA samples following CD24 forced expression in HCT116 **(A)** and knock down in SW620 **(B)** did not show a significant change in the expression level of Cten (p=0.1548 and p=0.1056, respectively) compared to the control EV or Luc. The quantity of Cten mRNA in each cell line was normalised to the expression of housekeeping HPRT1 in each cell line. Each experiment was conducted in triplicate and the data was analysed using Prism software.

4.2.1.6 Investigation of the Likelihood of Stabilising Cten Protein by CD24

The data from the quantification of the Cten mRNA were not surprising since CD24 and Cten appeared to have an effect on each other and it would be more likely that this would be a mutual protein stabilization rather than mutual gene induction. The ability of CD24 to stabilise Cten was studied using the cycloheximide (CHX) assy. CHX inhibits protein synthesis by blocking the elongation of the peptide during translation and thus treating cells with CHX allows the degradation of a protein to be followed. HCT116 cells (which express low levels of CD24 and low levels of CD24) were transfected with CD24 and Cten expressing plasmids, followed by treatment with CHX reagent 24 hrs after transfection. In the cells in which Cten was forcibly expressed along with co-transfection of the empty CD24 vector (EV/Cten+), both the ectopic Cten protein and the endogenous CD24 protein could be seen to undergo degradation (Figure 4-7, A). When cells were co-transfected with the CD24 and the Cten expression vectors (CD24+/Cten+), this resulted in a pronounced stabilisation of the Cten protein (Figure 4-7, B). For further validation, SW620 cells were transfected with either CD24 siRNA or luciferase control followed by CHX treatment 24 hrs after transfection. Expression of Cten was found to be stabilised by CD24 in cells treated with control siRNA (luciferase) and CHX (Figure 4-7, C) while degradation in the expression level of Cten was observed when CD24 was depleted from the cells (Figure 4-7, D). Taken together, these data strongly suggest that Cten is positively regulated by CD24 and that this is mediated through protein stabilization.

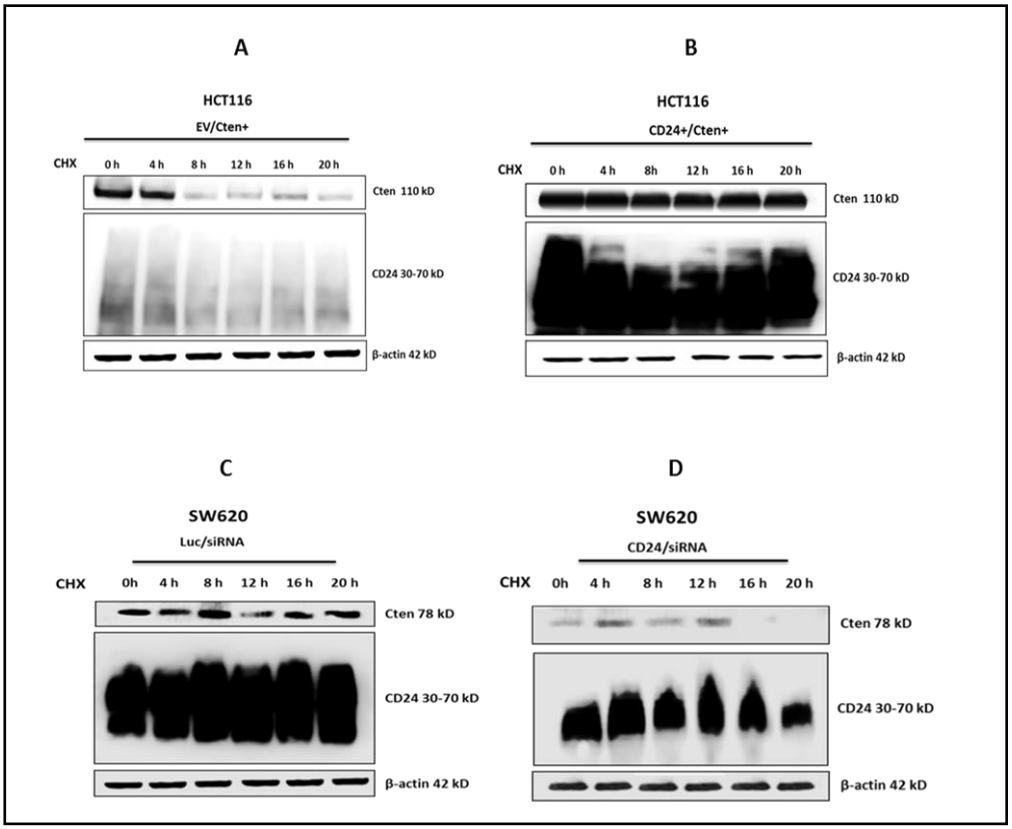


Figure 4-7. Validating the relationship between CD24 and Cten in CRC cell lines using cycloheximide (CHX) assay. HCT116 cells were transfected with CD24/Cten plasmids followed by treatment with CHX reagent 24 hrs after transfection. This resulted in a stabilisation of Cten protein expression **(B)**, while Cten expression level degraded in HCT116 cells that were treated with EV and Cten plasmid together, followed by CHX treatment **(A)**. SW620 cells were treated with CD24/siRNA and CHX 24 hrs later. This resulted in degradation in the expression level of Cten **(D)**, but Cten expression was found to be stabilised by CD24 in cells treated with luciferase and CHX **(C)**. The loading control (β-actin) showed the amount of proteins loaded on the gel (25 µg).

4.2.1.7 Investigation of CD24-Cten Interaction Using Co-IP

Previous data revealed a mutual effect on protein levels between CD24 and Cten and suggested that this may be through protein stabilization. Therefore, we sought to next explore whether CD24 and Cten might physically interact with each other. A protein complex immunoprecipitation (Co-IP) assay was performed in the SW620 cell line and an interaction between CD24 and Cten molecules was evaluated by immunoblotting.

CD24 co-immunoprecipitated with Cten in SW620. Samples were precleared with beads, input used as a positive control, and beads as a negative control. Anti-CD24 SWA11 (1:2000) antibody was used to detect if CD24 and Cten molecules interact in a protein complex (Figure 4-8). It is apparent that Cten binds to CD24 detected by Anti-CD24 SWA11 Ab. A band seen on the beads' lane is an artefact that might have been produced due to prolonged exposure or because of Anti-CD24 antibody binding to the blocking agent. The blot showed that both molecules may interact with each other in a complex protein.

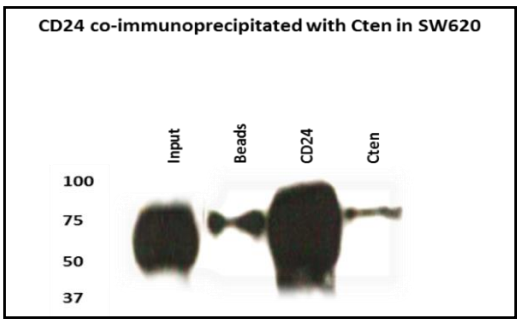


Figure 4-8. CD24 co-immunoprecipitated with Cten in SW620. Samples were precleared with beads, input used as a positive control, and beads as a negative control. Anti-CD24 SWA11 (1:2000) antibody was used to detect if CD24 and Cten molecules interact in a protein complex. It is apparent that Cten binds to CD24 detected by Anti-CD24 SWA11 Ab. A band seen on the beads' lane is an artefact that might have been produced due to prolonged exposure or because of Anti-CD24 antibody binding to the blocking agent. The blot showed that both molecules may interact with each other in a complex protein.

4.2.1.8 Investigation of the Effect of Cten Knockout on the Expression of FAK and ILK

Our data showed that CD24 positively regulates Cten, ILK and FAK. Furthermore it was probably regulating Cten through post-transcriptional stabilization. Previous data from the lab had suggested that Cten can also regulate ILK and FAK thus raising the question of whether CD24 and Cten acted independently on ILK/FAK or whether there was a pathway through from CD24 via Cten to ILK/FAK. A variant of the SW620 cell line in which Cten had been deleted ($SW620^{CtenKO}$) using clustered regularly interspaced short palindromic repeats–associated nuclease 9 (CRISPR-Cas9) genome editing was available for testing. This had been created by other PhD students in our group.

This cell line shows high CD24 and absent Cten (Appendices Figure 9-4) therefore allowing the Cten-dependent targets to be tested. Cten knockout was evaluated by Western blot and qPCR prior to any forthcoming experimental work. qPCR for Cten mRNA levels after Cten knockout illustrated a significant reduction in Cten expression level ($p = 0.0009$) compared to the control ($SW620^{WT}$) (Figure 4-9, B) representing the successful Cten gene mutation by CRISPR. The data generated using Western blotting confirmed loss of Cten protein expression. The Western blots also showed that knocking out of Cten has resulted in a complete depletion in the expression levels of FAK and ILK proteins. These findings demonstrate that Cten is a direct target of CD24 where cell signals can pass through to regulate FAK and ILK (Figure 4-9, A).

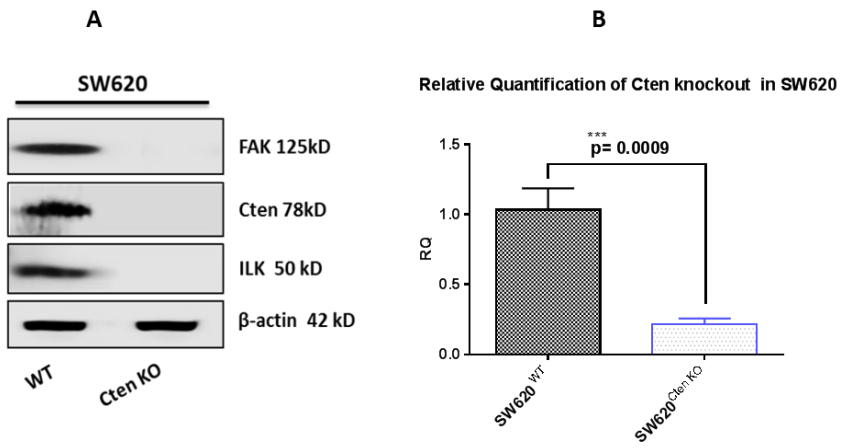


Figure 4-9. Cten knockout in SW620 using CRISPR technique. (A) Cten was knocked out in SW620 cells using CRISPR-Cas9. The Western blot results showed that the expression level of FAK and ILK were nullified following Cten knockout. (**B)** qPCR for Cten mRNA levels after Cten knockout show a significant reduction in Cten expression level ($p < 0.05$) in comparison with the control ($SW620^{WT}$).

4.2.1.9 Investigation of the Effect of Cten Knockout on Cell Function

Since CD24 has been found to regulate cell functions including cell motility and invasion as shown in chapter 3, we hypothesised that these functions could possibly be mediated through Cten which is up-regulated by CD24. To test this hypothesis, Transwell migration and Matrigel invasion assays were carried out in $SW620^{CtenKO}$ which has been confirmed as not having Cten, but as being a high expressor of CD24 (as confirmed using Western blot and qPCR see Appendices Fig. 9-4). The data from the functional assays demonstrated that knocking out of Cten led to a significant reduction in both Transwell cell migration and invasion ($p < 0.0001$, Figure 4-10, A & B).

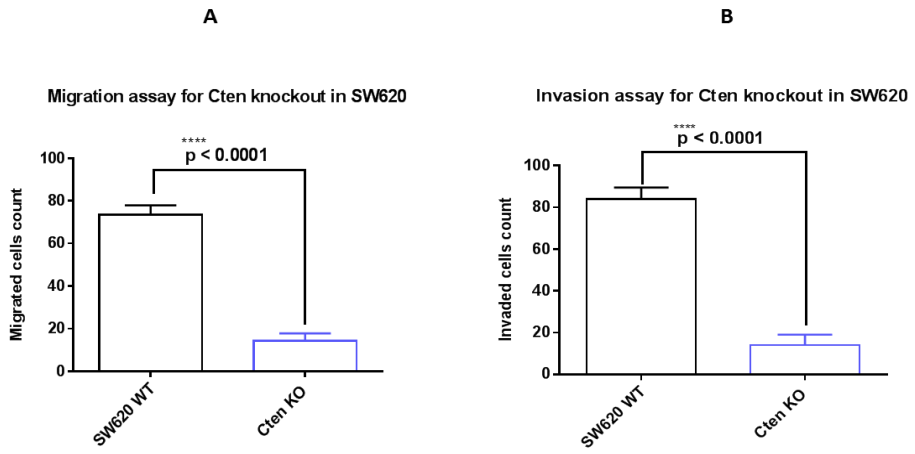


Figure 4-10. Functional assays following Cten knockout in CRC. Transwell migration **(A)** and invasion **(B)** assays were performed in $SW620^{CtenKO}$ and the control ($SW620^{WT}$) cells. A significant reduction in both cell migration and invasion ($p < 0.0001$) was observed compared with the control (Luc), demonstrating the importance of Cten in regulating cell functions.

4.2.1.10 Investigation of Likely Association between CD24 and other Adhesion Molecules

Our studies have shown that CD24 regulates cell motility and part of this may be through focal adhesions which form when integrin molecules cluster together. Further studies to investigate the relationship between CD24 and other proteins involved in signal transduction and localised near focal adhesions were conducted. Possible alterations in the expression levels of four vital proteins (i.e., Vinculin, β-catenin, Paxillin, and Src) that have been reported to interact with some focal adhesion-associated molecules such as FAK or considered as actin binding proteins (Quadri, 2012) were assessed after knocking down CD24 in the SW620 cell line. Western blot data illustrated that down-regulation of CD24 caused a reduction in the expression level of Src; an augmentation in the expression level of β-catenin, while no change in the expression levels of Paxillin and Vinculin was observed (Figure 4-11). These findings indicate Src as a further potential downstream target of CD24.

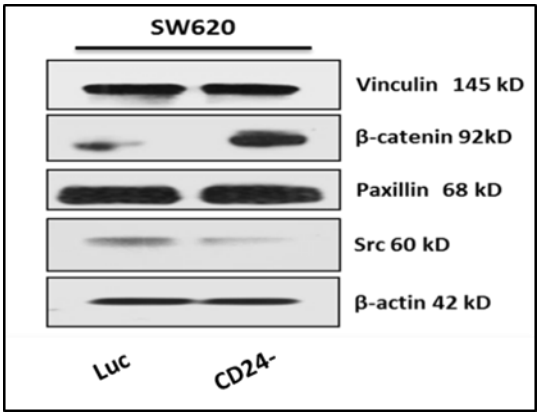


Figure 4-11. Investigation of the relationship between CD24 and other focal adhesion-associated molecules. Knockdown of CD24 led to a reduction of the expression level of Src, but an up-regulation of the expression level of β-catenin, and no change occurred in the expression levels of Vinculin and Paxillin. The loading control (β-actin) showed the amount of proteins loaded on the gel (25 µg).

4.2.1.11 Investigation of the Association between CD24 and EMT Markers in CRC

Epithelial-mesenchymal transition (EMT) is considered to be a significant event in the development of tumour metastasis. It results in acquisition of mesenchymal features in epithelial cells and is also associated with increase in cell motility. We investigated whether CD24 could be involved in EMT. Molecules used as markers of EMT such as E-cadherin, N-cadherin and Snail were evaluated following CD24 manipulation in HCT116 and SW620 using Western blot. The data illustrated that CD24 up-regulates N-cadherin and down-regulated E-cadherin, also called "cadherin switch", (Figure 4-12, A & B).

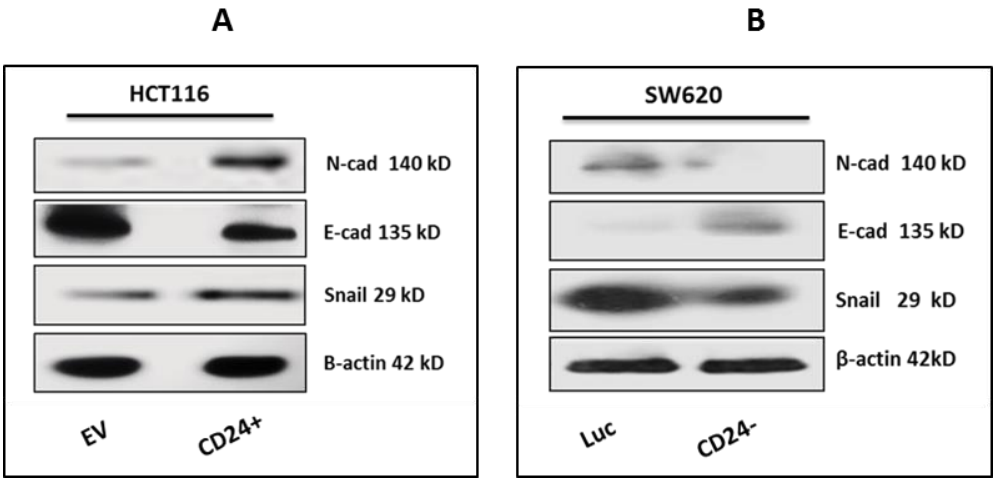


Figure 4-12. Investigation of the relationship between CD24 and EMT markers in CRC. Forced expression of CD24 in HCT116 **(A)** and down-regulation of CD24 in SW620 **(B)** indicated that CD24 up-regulates N-cadherin and Snail, but down-regulates E-cadherin in both cell lines. The loading control (β-actin) showed the amount of proteins loaded on the gel (25 µg).

4.2.1.12 Investigation of the Role of CD24 in Tumour Formation

4.2.1.13 Relationship between CD24 and Stemness in CRC

Once a tumour cell has migrated to a new site, it must establish itself and grow at that site. We have shown that CD24 confers features of stemness on cells and thus we sought to define the pathway through which this may be mediated.

Bcl-2 and Bcl-XL are two homologous molecules which are usually associated with inhibition of apoptosis. In the normal colon, however, Bcl2 is usually located in the base of the crypt in the stem cell compartment. Bcl-XL is often up-regulated in CRC due to amplification in 20q and it may provide some of the same functions as Bcl-2 (especially since Bcl-2 is frequently reduced due to loss of chromosome 18q). Expression of Bcl-2 and Bcl-XL was evaluated following CD24 manipulation in RKO (forced expression) and SW620 (knockdown) CRC cell lines. No change in the expression levels of either of the molecules was

observed, indicating that these molecules are not targets of CD24 (Figure 4-13, A & B).

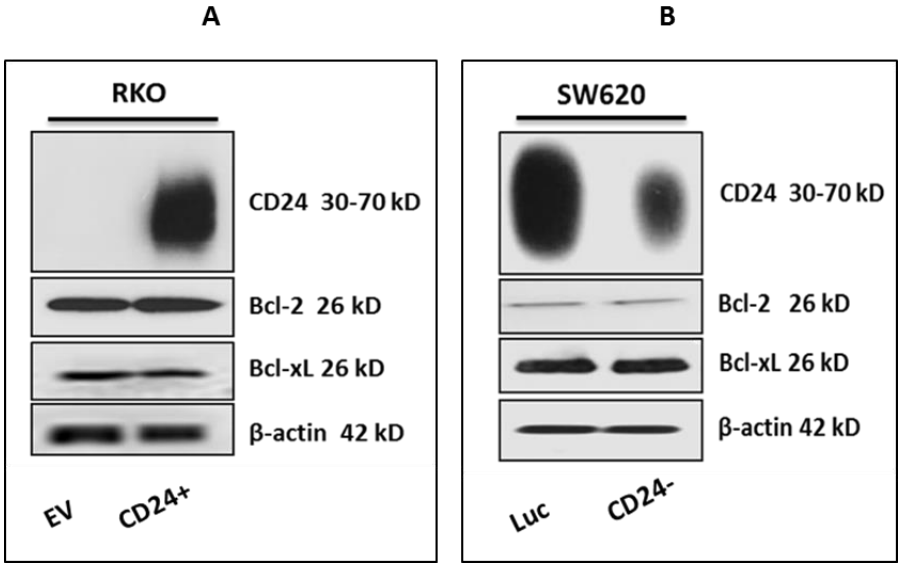


Figure 4-13. Investigation of the relationship between CD24 and apoptosis-related molecules in CRC. Bcl-2 and Bcl-XL were evaluated following CD24 manipulation in RKO **(A)** and SW620 **(B).** No change in the expression level of both proteins was found. The loading control (β-actin) showed the amount of proteins loaded on the gel (25 µg).

4.2.1.14 Investigation of the Relationship between CD24 and AKT in CRC

Previous data produced by our group (but not published) had suggested that AKT signalling may be a target of CD24 signalling. Using a phsophokinome array, CD24 was shown to cause activation of AKT as well as various AKT downstream targets such as phosphorylation of GSK-3α/ß at Serine-9, mTOR at ser-2448 (a main activation site in mTOR downstream to AKT), phosphorylation of p27 at T198, c- Jun, and CREB (cAMP response element-binding protein) (AHMED, 2011).

In order to investigate whether CD24 could activate the AKT pathway to confer stemness, we down-regulated CD24 in two CD24-positive CRC cell lines, DLD-1 and GP2D. Both have constitutive AKT activation because of PIK3CA

mutations (Fadhil et al., 2010). The effect of CD24 modulation on AKT activation as well as on the activation / expression of a number of downstream targets of AKT was evaluated.

We noted that knockdown of CD24 in DLD-1 and GP2D cell lines resulted in no significant reduction in the expression level of p-AKT (Thr308 – activated by the mutant PIK3CA), while the expression level of p-AKT (Ser473) significantly decreased in both cell lines (Figure 4-14, A & B). No change was observed in the expression level of total AKT since Pan-AKT antibody (i.e., phosphorylated, and non-phosphorylated AKT) was used as a measure of total AKT protein level. These results are consistent with the preliminary results and confirm the effect of CD24 on AKT activation at Ser473.

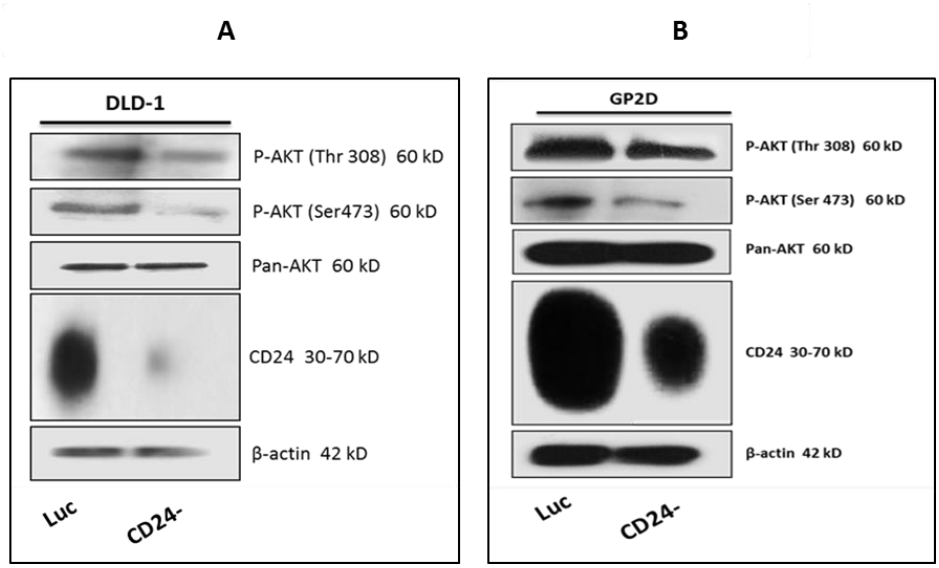


Figure 4-14. **Investigation of the relationship between CD24 and PI3K/AKT in CRC.** CD24 was knocked down in DLD-1 **(A)** and GP2D **(B)** using CD24 siRNA. Western blot was used to evaluate possible changes in total AKT and phosphorylated AKT afterwards. Only a minor reduction was recorded in the expression level of p-AKT (Thr308) in both cell lines, while a significant decrease in the expression level of p-AKT (Ser473) was observed. No modification in the expression level of total AKT protein control (Pan-AKT) was found. Fifty µg of proteins were used for the detection of the phosphorylated forms of proteins while 30 µg was used in the case of the whole protein.

The phosphokinome array previously carried out following CD24 knockdown in CRC cells illustrated that several downstream targets of the AKT pathway were functionally activated by CD24 such as phospho-FAK (p-FAK), phospho-mammalian target of rapamycin (p-mTOR), phospho-Endothelial NOS (p-eNOS), phospho-cAMP responsive element binding protein (p-CREB), phospho-c-Jun(p-c-Jun), phospho-p27 (p-p27) and others. We evaluated the expression level of these molecules following CD24 knockdown in DLD-1, and we found consistent results with those shown in the array especially in p-FAK, P-eNOS and p-CREB. The anti-p-mTOR and dp-P27 and c-Jun antibodies did not work (Figure 4-15). These data together with the preliminary data demonstrate that CD24 is a likely activator of AKT pathway signalling.

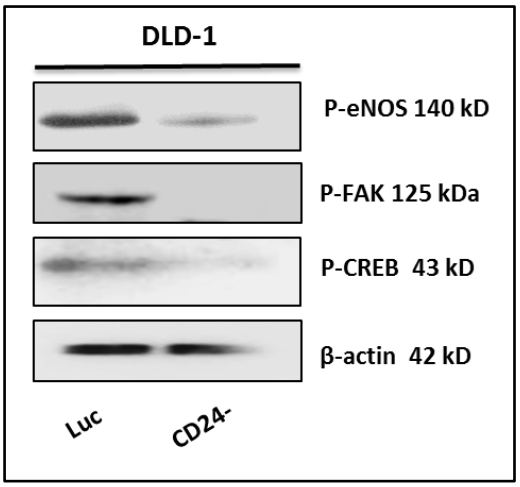


Figure 4-15. Investigation of the effect of CD24 on AKT downstream effects. To further validate the results obtained from the array experiment antibodies against the phosphorylated proteins were used in addition to antibodies against the whole proteins. CD24 knockdown resulted in deactivation of p-eNOS, p-FAK, and p-CREB. Fifty µg of proteins were used for the detection of the phosphorylated forms of proteins as well as the loading control.

4.2.1.15 Possible Use of Targeting CD24 and AKT in CRC

It was of interest that CD24 activity was targeted to Ser473 whilst Thr308 was a target of the PI3K signalling pathway. This prompted us to wonder whether CD24/PI3K could be targeted in combination as an alternative method for treating cancer cells. The PI3 kinase pathway can be targeted using the inhibitor LY294002 and we hypothesised that combining this with inhibition of CD24 would enhance the response. We therefore tested the cells (a) using two different concentrations of LY294002 (i.e. 10 μM and 50 μM), (b) using CD24 siRNA, or (c) both PI3 kinase inhibitor LY294002 and CD24 siRNA together. DMSO (the vehicle of LY294002) and non-targeting siRNA were used as controls, respectively. The cells of GP2D and DLD-1 were grown for 24 hrs in a serum-free medium before treatment with LY294002 at 10 μM and 50 μM following cell transfection with CD24 siRNA or non-targeting siRNA control. Activation of AKT was evaluated by Western blot.

The data showed that treatment with LY294002 10 μM alone resulted in a change in the activation of AKT at Ser473 residue especially in DLD-1 cells, but more reduction in the activation of AKT at the Thr308 residue was observed in both cell lines. We also detected that treating cells with LY294002 alone at 50 μM caused a reduction in the AKT activation at both residues almost similar to the effect of LY294002 at 10 μM concentration. In contrast, down-regulated CD24 did not show an effect on the activation of AKT at Thr308 residue, but a reduction in its activation at the Ser473 residue was observed. Combination of CD24 siRNA treatments (Figure 4-16, B) with the PI3 kinase inhibitor LY294002 resulted in a reduction in the activation of AKT at both residues: AKT Ser473 and AKT Thr308. The data also showed that combination of CD24

siRNA with the lower dose of the AKT inhibitor, had a greater effect than the higher dose of the AKT inhibitor when used alone. Together these results show evidence that CD24 works with PI3K to fully activate AKT and combined therapy may allow reduction in the dose of the PI3K inhibitor which is required.

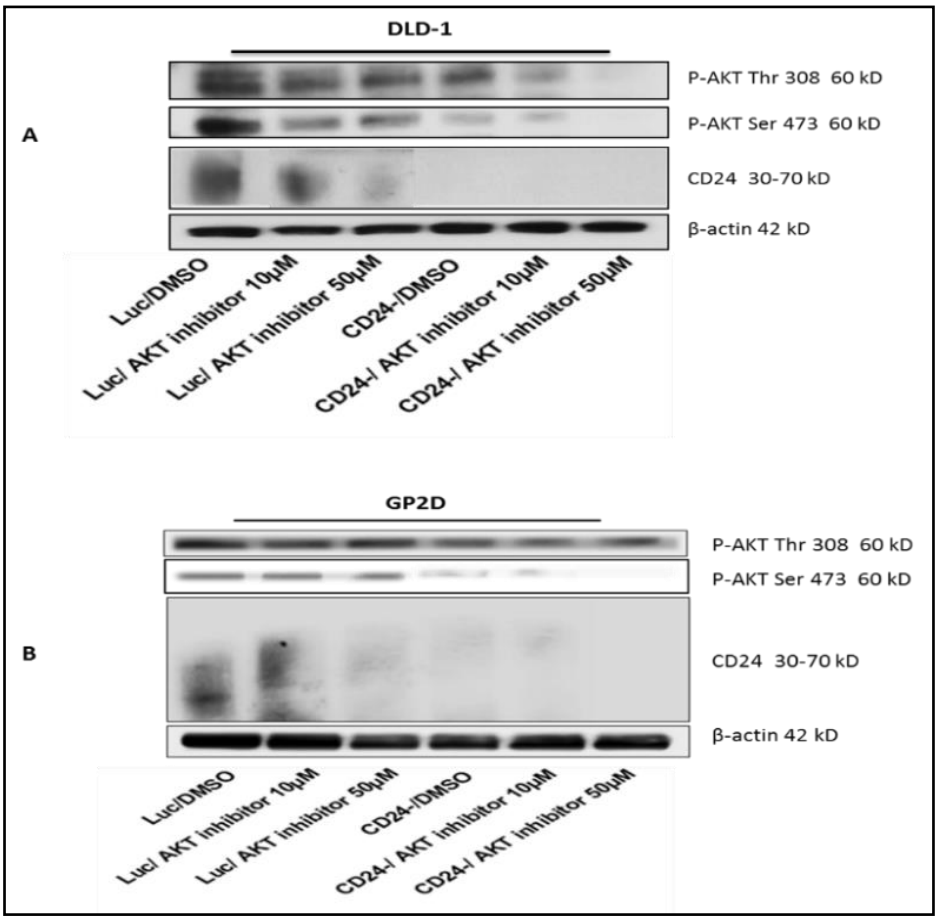


Figure 4-16. Validation of a relationship between CD24 and phosphorylated AKT using CD24 siRNA and AKT inhibitor. Two CRC cell lines (DLD-1 and GP2D) were used to study the possible association between CD24 and the phosphorylation AKT at two sites: Thr308 and Ser473. CD24 siRNA and AKT inhibitor at 10 μM and 50 μM were used to transfect the cells separately or in combination. Western blot was used to evaluate P-AKT at both sites. In both cell lines **(A & B),** the LY294002 at 10 μM along with CD24 siRNA reduced the activation of AKT at Ser473 and Thr308, while LY294002 at 10 μM alone reduced the activation of AKT at Ser473, but not at Thr308. In contrast to LY294002, CD24 siRNA had an opposite effect: the activation of AKT at Ser473, but not at Thr308. Treating cells with LY294002 alone at 50 μM led to a reduction in the AKT activation at both sites corresponding to the effect of a combined treatment of LY294002 at 10 μM and CD24 siRNA. CD24 siRNA together with AKT inhibitor at 50 μM has led to a complete depletion of CD24 and p-AKT Ser473.

4.2.1.16 Investigation of the Effect of CD24 siRNA/ AKT Inhibitor on Cell Functions in CRC

Although the AKT pathway is associated with cell survival and stemness (Ji and Wang, 2012, Ma et al., 2008), it has also been associated with cell motility and invasion (Kim et al., 2001, Yoeli-Lerner and Toker, 2006). Functional studies including cell Transwell migration, Matrigel invasion and PrestoBlue proliferation were carried out following the transfecting of DLD-1 and GP2D cells with LY294002 AKT inhibitor at 10 μM and 50 μM or CD24 siRNA or in a combination between these treatments. We observed that both cell migration and invasion reduced significantly following treatment of the cells with LY294002 at 10 μM and 50 μM as well as following treatment with CD24 siRNA. However, we found that treatment of the cells with CD24 siRNA together with LY294002 at 10 μM resulted in a significant reduction in cell migration and invasion in comparison with the effect of each treatment added to the cells separately ($p < 0.01$). Remarkably, the effect of combined CD24 siRNA and LY294002 at 10 μM showed a similar effect on cell migration and invasion to a 50 μM concentration of LY294002 (Figure 4-17).

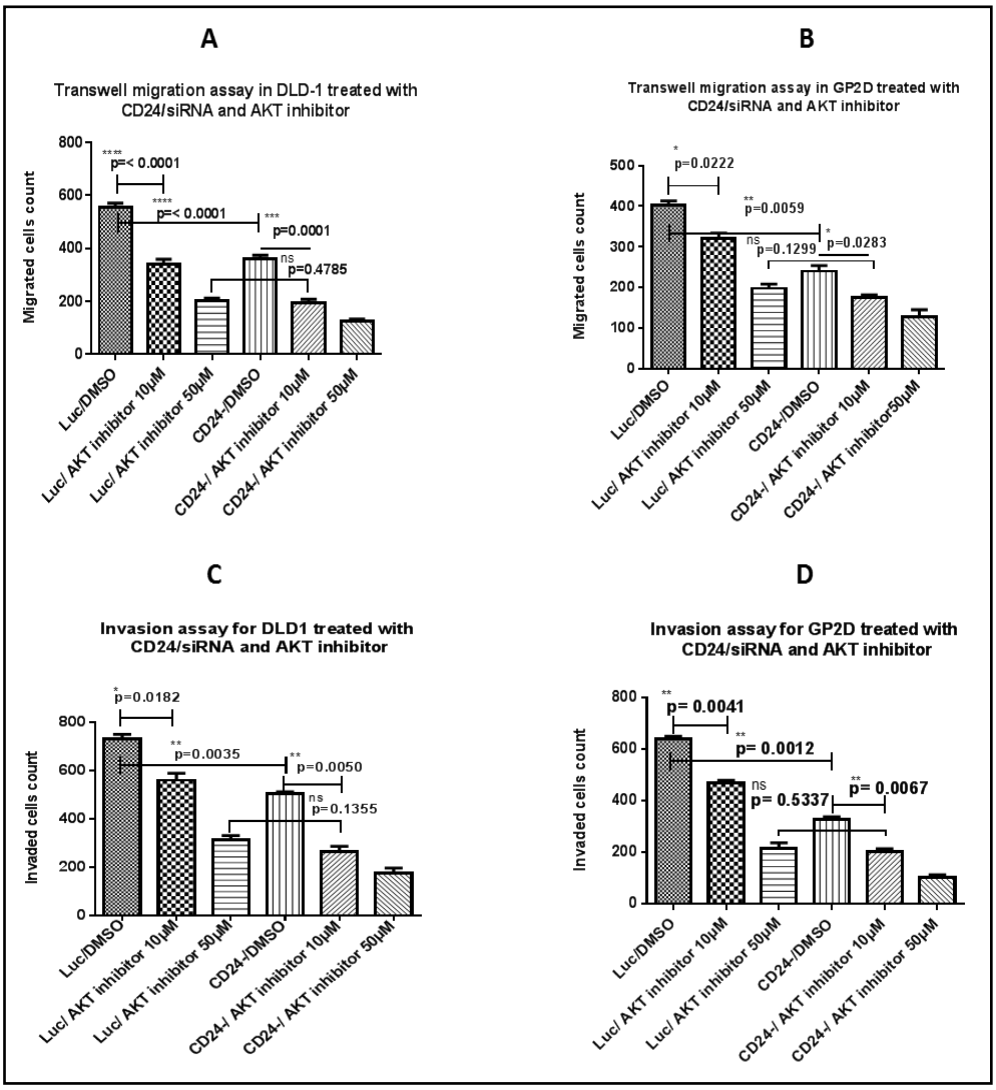


Figure 4-17. Functional assays for DLD-1 and GP2D cells treated with CD24 siRNA and AKT inhibitor. Transwell migration assay **(A & B).** Matrigel invasion assays **(C&D).** Cells treated with CD24 siRNA resulted in significant decrease in cell migration/invasion in comparison to the control (Luc/DMSO). A reduction in the number of migrated/invaded cells was also observed when the cells were treated with AKT inhibitor 10 μM, but more significant reduction was found after treating cells with 50 μM of AKT inhibitor. A similar effect was observed after combining CD24 siRNA and AKT inhibitors at 10 μM or 50 μM respectively. Cell migration and invasion depletion level after treating cells with CD24 siRNA accompanied with 10 μM of AKT inhibitor was found to be almost the same as their levels after treating cells with AKT inhibitor 50 μM, indicating the importance of such correlation between CD24 and AKT in enhancing cell migration and invasion in CRC.

As it can be seen from Figure 4-18, cells treated with CD24 siRNA demonstrated a significant decrease in the cell proliferation compared to the control (Luc/DMSO). A reduction in cell proliferation was also observed when the cells were treated with AKT inhibitor at 10 µM concentration, but a more marked reduction was seen after treating cells with 50 µM of AKT inhibitor. A comparable effect was detected after combining CD24 siRNA and AKT inhibitor at 10 µM or 50 µM, respectively. Interestingly, treating cells with CD24 siRNA accompanied with 10 µM of AKT inhibitor has been found to have a similar effect to that recorded after treating cells with AKT inhibitor 50 µM alone, suggesting that such a correlation between CD24 and AKT is enhancing cell proliferation in CRC.

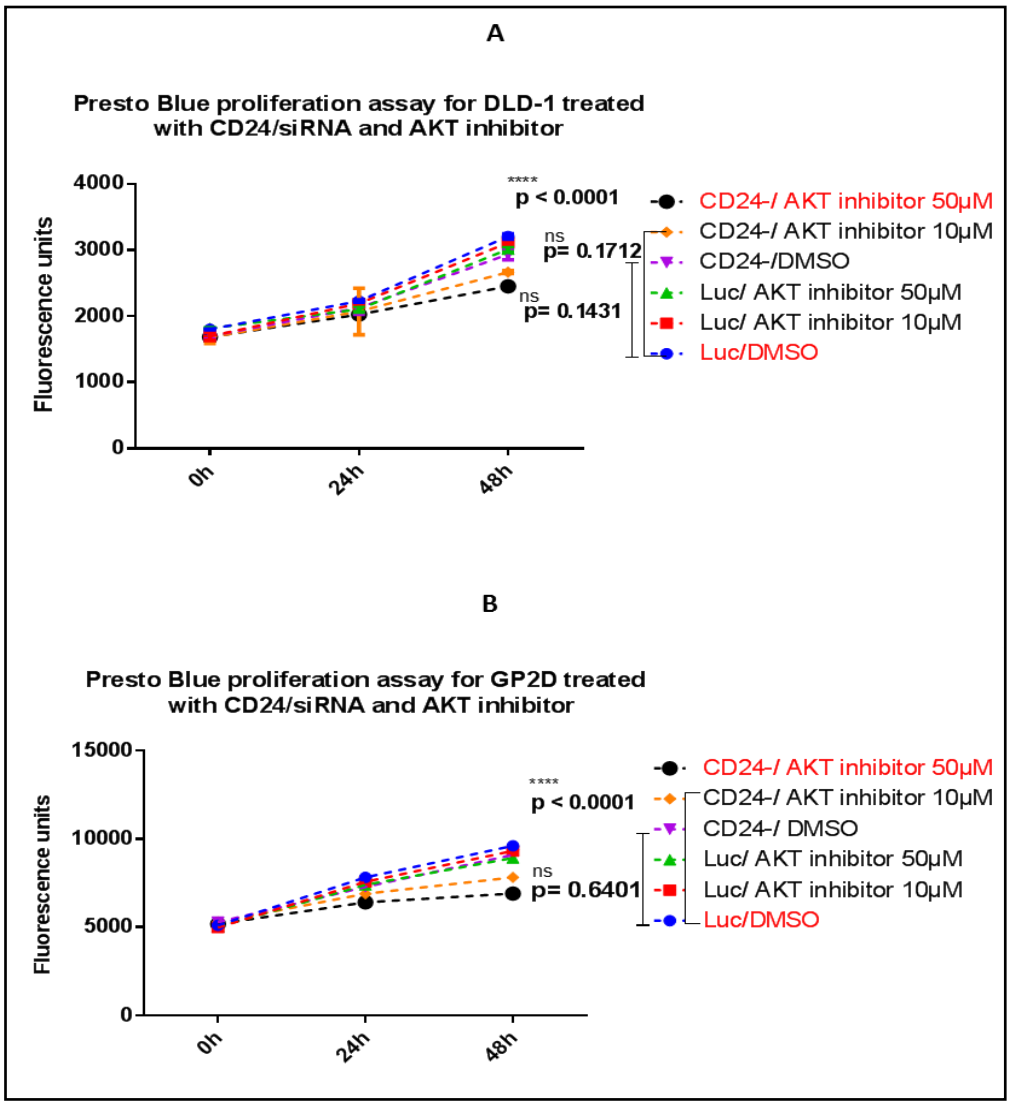


Figure 4-18. Investigation of the effect of CD24 siRNA/ AKT inhibitor on cell proliferation in DLD-1 and GP2D. PrestoBlue proliferation assay was performed following transfection of DLD-1 **(A)** and GP2D **(B)** cells with CD24 siRNA or AKT inhibitor. Cells treated with CD24 siRNA did not show any significant reduction in cell proliferation compared to the control (Luc/DMSO). A reduction in cell proliferation was also observed following the addition of AKT inhibitor at 10 µM concentration to the cells, a further significant reduction was observed with 50 µM of AKT inhibitor. Similar observations were made after combining CD24 siRNA and AKT inhibitor at 10 µM or 50 µM, respectively. Treating cells with CD24 siRNA accompanied with 10 µM of AKT inhibitor gave the same effect as treating cells with AKT inhibitor 50 µM. The assay was repeated three times and the data were analysed using Prism Pad 6 software (Two-way ANOVA).

4.2.2 Investigation of CD24 Downstream Targets in Pancreatic Cancer

The similarity of CD24 function in CRC and pancreatic cancer have been shown in our previous data. Therefore, we attempted to investigate the potential downstream targets of CD24 in pancreatic cancer as it seems to be an ideal model with which to validate our observations found in CRC. Forced ectopic expression of CD24 in a CD24-lower expressor pancreatic cancer cell line (Panc-1), using pcDNA3.1 empty vector and pcDNA3.1-CD24 plasmids and knockdown of CD24 in other CD24-higher expressor pancreatic cancer cell lines, PSN-1 and COLO357**,** using CD24 siRNA and luciferase siRNA as a control (Luc) were performed, followed by immunoblotting to evaluate such mirrored changes in the expression levels of the proteins of interest (i.e., Cten and its downstream targets).

Western blot data (Figure 4-19, A) demonstrated that CD24 overexpression in the Panc-1 cell line resulted in an augmentation in the expression level of Cten and its downstream targets (FAK and ILK). Likewise, down-regulation of CD24 in PSN-1 and COLO357 led to a reduction in the expression levels of Cten, FAK and ILK (Figure 4-19, B & C). Such a consistency between these data and the previous ones found for CRC indicate the dynamic role of CD24 in regulating cell functions in these tumours by signalling through Cten and its downstream targets.

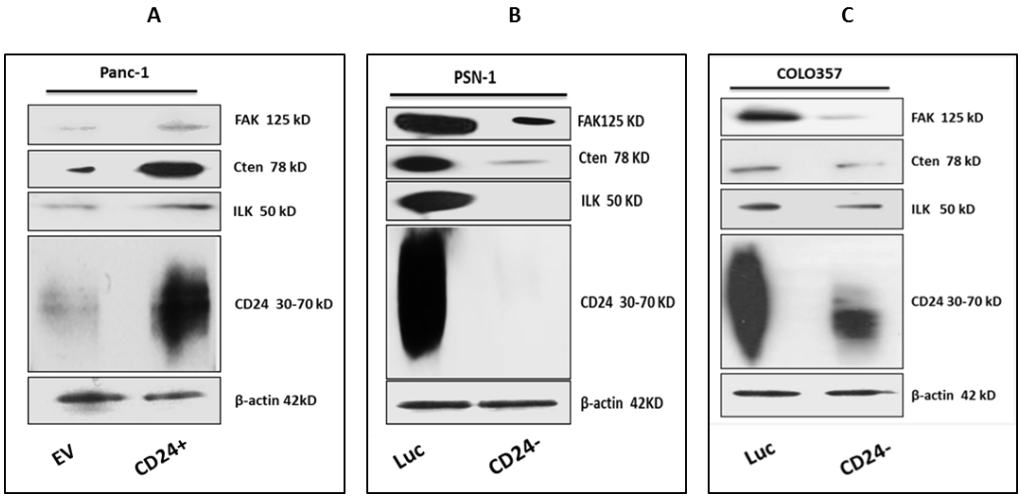


Figure 4-19. Investigation of CD24 downstream targets in pancreatic cancer cell lines. CD24 was forcibly expressed in Panc-1 using pcDNA3.1plasmid and an empty vector was used as a control. CD24 was knocked down in PSN-1 and COLO357 using CD24 siRNA and non-sense siRNA as a control (Luc). Western blot data **(A)** showed that CD24 up-regulation in Panc-1 cell led to an increase in the expression level of Cten, FAK and ILK. In contrast, CD24 down-regulation in PSN-1 and COLO357 caused a decrease in the expression level of Cten, FAK and ILK **(B & C)**. The loading control protein (β-actin) showed the amount of proteins loaded on the gel (25 μg). All the experiments were done in triplicate.

4.2.2.1 Investigation of the Regulation of Cten by CD24 in Pancreatic Cancer

The expression of Cten at mRNA was evaluated using qPCR in pancreatic cell lines in order to determine whether Cten expression is regulated by CD24 at mRNA level following up-regulation of CD24 in Panc-1 and down-regulation of CD24 in PSN-1. No significant alteration was observed in the Cten mRNA levels in RNA samples extracted after each transfection in the above cell lines ($p = 0.291$ and $p = 0.098$, respectively, Figure 4-20, A & B). This finding is consistent with that observed in CRC cell lines signifying that CD24 regulates Cten at the protein level but not at mRNA level.

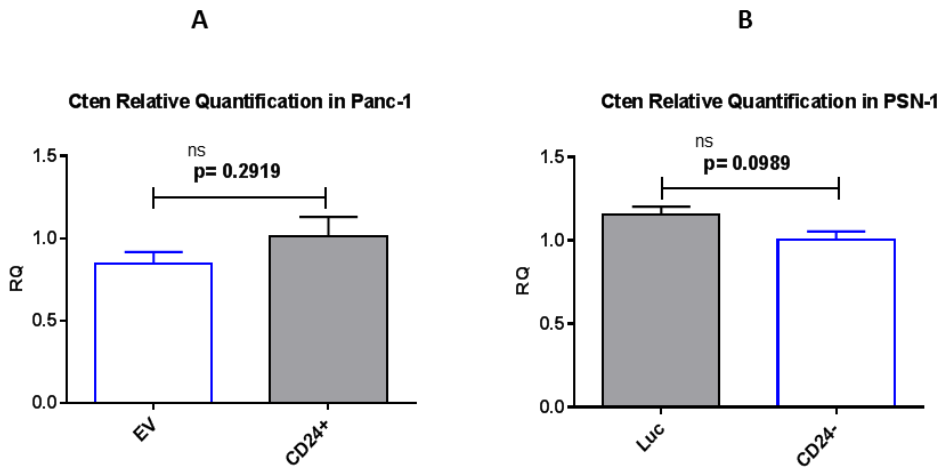


Figure 4-20. Investigation of the effect of CD24 on Cten mRNA in pancreatic cancer. CD24 was forcibly expressed in Panc-1 and knocked down in PSN-1. The qPCR for Cten mRNA levels in RNA samples extracted after each transfection did not show significant changes in Cten mRNA expression levels (p=0.29 and p=0.098, respectively) compared to the control (EV or Luc). The quantity of Cten mRNA in each cell line was normalised to the expression of housekeeping HPRT1 in each cell line. Each experiment was conducted in triplicate. The data was analysed using Prism software.

4.2.2.2 Investigation of the Likelihood of Stabilising Cten Protein by CD24 in Pancreatic Cancer

Since Cten was found to be stabilised by CD24 at protein level in CRC cell lines, as confirmed by CHX assay, we wanted to further confirm these findings using pancreatic cell lines. PSN-1 cells were transfected with CD24 siRNA followed by CHX treatment 24 hrs later. The data demonstrated that a degradation in the expression level of Cten occurred (Figure 4-21, B), while Cten expression was stabilised by CD24 at protein level in the same cells following a treatment with control siRNA (luciferase) and CHX (Figure 4-21, A). These data match with previous observations of CRC and confirm the notion that Cten is targeted by CD24.

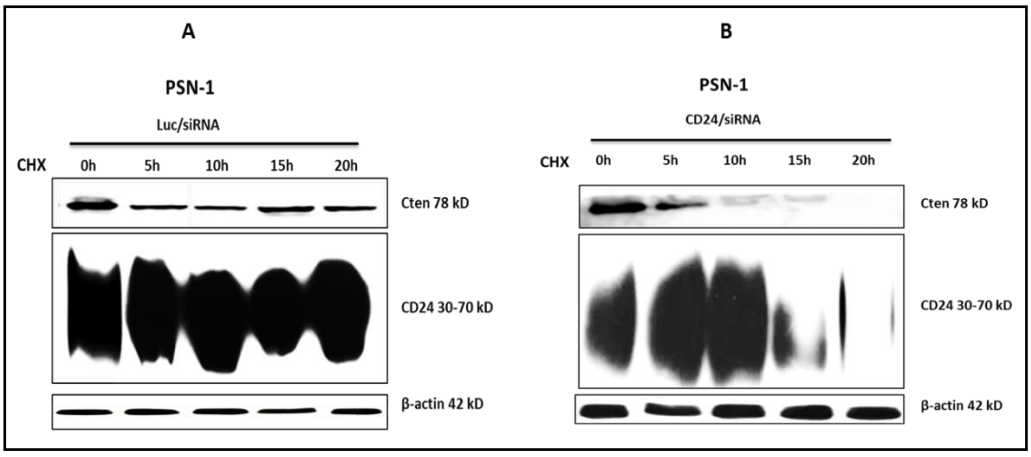


Figure 4-21. Investigation of Cten stabilisation by CD24 in pancreatic cancer using CHX assay. PSN-1 cells were transfected with CD24 siRNA and non-sense siRNA (luciferase), then treated with CHX 24 hrs later in both conditions. A degradation in the expression level of Cten was observed following CD24 knockdown and CHX **(B)**, while Cten expression was found to be stabilised by CD24 in cells treated with luciferase and CHX **(A)**. The loading control (β-actin) showed the amount of proteins loaded on the gel (25 µg).

4.2.2.3 Investigation of the Association between CD24 and other Related Focal Adhesion Molecules in Pancreatic Cancer

The main aim of these investigations was to identify whether CD24 is associated with EMT markers that have a significant role in the induction of cancer cells leading to acquisition of invasive and metastatic processes and enhancement of cell functions in pancreatic cancer. Consequently, CD24 was forcibly expressed in Panc-1 cells and the changes in the expression of CD24 or other proteins of interest were evaluated using Western blot. We observed that the expression level of Src increased following up-regulation of CD24 in Panc-1, while just a slight increase was seen in the excretion level of Vinculin, and no change was observed in the expression of Vimentin (Figure 4-22, A). These results are consistent with that seen in CRC.

We tested the possible correlation between CD24 and EMT markers in pancreatic cancer. Changes in the expression levels of EMT markers (E-cadherin, N-cadherin, and Snail) were evaluated following CD24 overexpression in Panc-1. We found that CD24 causes an increase in the expression level of N-cadherin and Snail, but a reduction in the expression level of E-cadherin (Figure 4-22, B). Together these data show that CD24 up-regulates N-cadherin and Snail, but it down-regulates E-cadherin in pancreatic cancer.

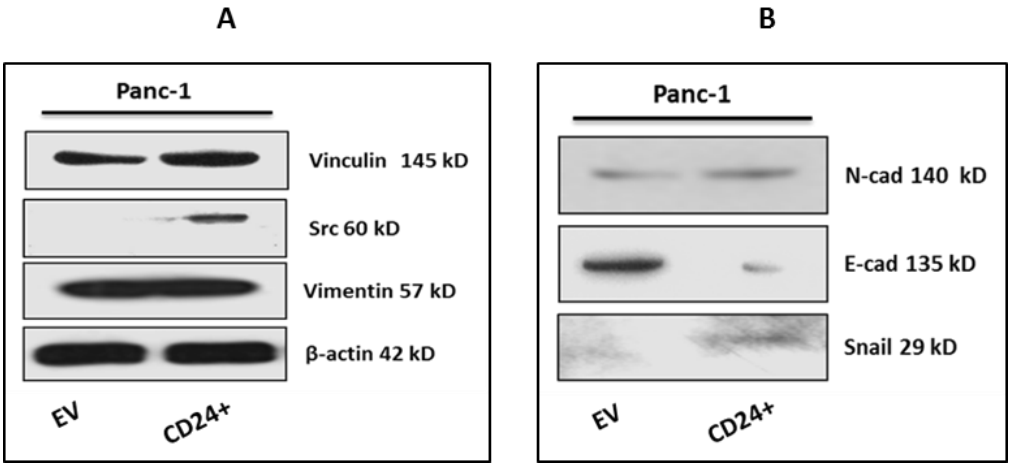


Figure 4-22. Investigation of the association between CD24 and focal adhesion molecules, and EMT markers in pancreatic cancer cell line. CD24 forced expression was performed in Panc-1 cell line. **(A)** Possible changes in the expression of CD24 were evaluated using Western blot. The expression level of Src increased, a slight but not significant increase was found in Vinculin expression, while no change was found in the expression of Vimentin following CD24 overexpression. **(B)** The expression level of N-cadherin and Snail increased because of up-regulation of CD24 in Panc-1, while a reduction in the expression level of E-cadherin was detected. The loading control (β-actin) showed the amount of proteins loaded on the gel (25 µg).

4.2.3 Investigation of CD24 Downstream Targets in HCC

The similarity of CD24 function in CRC and pancreatic cancer have been shown in our previous data. As far as we know, no studies have completely evaluated the correlation between CD24 and Cten and their significant role in HCC. CD24 was knocked down in a CD24-higher expressor liver cancer cell line (HUH7) using specific siRNA, and luciferase siRNA, was used as a control. The modifications in the expression levels of Cten and its downstream targets (FAK and ILK) were evaluated using the Western blot test. We noticed that CD24 down-regulation resulted in a reduction in the expression level of Cten as well as FAK and ILK (Figure 4-23, A). No other liver cancer cell lines were used to validate these findings. However, these data are highly consistent with earlier observations in CRC and pancreatic cancer.

In order to investigate the relationship between CD24 and EMT markers in HCC due to their significance in enhancing the metastatic potential of cancer cells as well as inducing changes in cellular phenotypes in cancer, the alterations in the expression levels of EMT markers following CD24 knockdown in HUH7 were evaluated using Western blot. Such a transfection resulted in a reduction in the expression levels of N-cadherin and Snail, but in an increase in the expression level of E-cadherin (Figure 4-23, B). This verifies that CD24 up-regulates N-cadherin and Snail, but it down-regulates E-cadherin in the three-cancer model that has been tested so far.

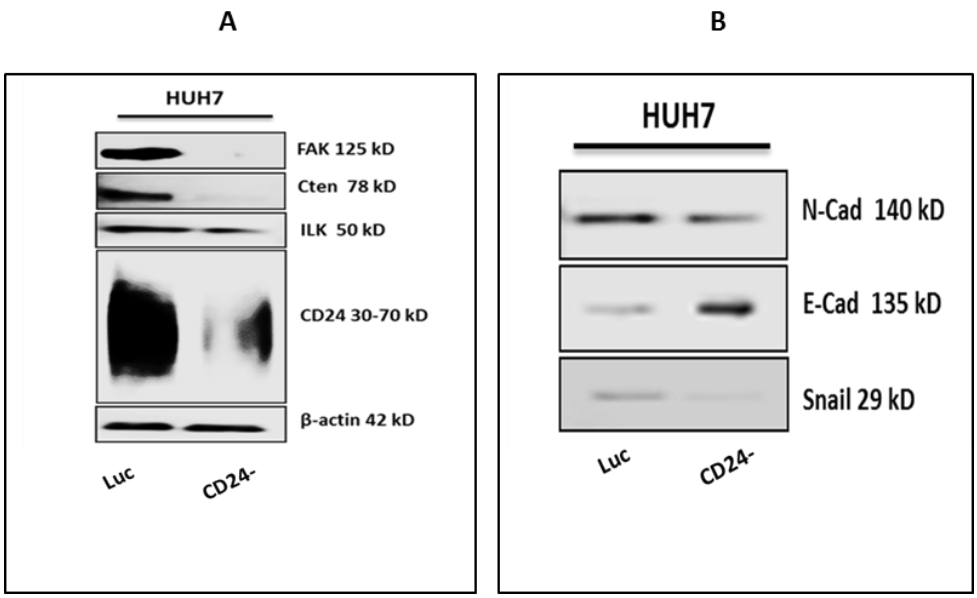


Figure 4-23. (A) Investigation of the CD24 downstream targets in liver cancer. CD24 was knocked down in HUH7 with a specific siRNA, luciferase was used as a control. Western blot data showed that CD24 knockdown has led to a significant decline in the expression level of Cten, FAK and ILK. The loading control protein (β-actin) showed the amount of proteins loaded on the gel (25 μg). **(B) Investigation of the relationship between CD24 and EMT markers in liver cancer.** Following CD24 overexpression in HUH7, a reduction in the expression levels of N-cadherin and Snail was detected, while a surge in the expression level of E-cadherin was found. All the experiments were done in triplicate.

4.2.4 Investigation of CD24 Downstream Targets in Non-Small-Cell Lung Cancer (NSCLC)

In attempts to identify the mechanism by which CD24 might regulate cell activities in lung cancer, we manipulated CD24 expression in three NSCLC cell lines by either overexpression of CD24 in low expressor cell lines, or down-regulation of CD24 in higher expressor cell lines using specific siRNA. The experimental validations were also performed with methods similarly to those used in previous investigations performed in CRC, pancreatic and liver cancer. The purpose of this study was to find likely downstream targets of CD24 in NSCLC and to detect whether CD24 might behave in this type of cancer

similarly to behaviours seen in the previous three-cancer model. The changes in the expression of conceivable downstream targets of CD24 were evaluated following overexpression of CD24 in H460 (CD24-low expressor) and knockdown of CD24 in H226 and A549 (CD24-high expressors) using Western blotting. Since data from the previous investigations of the biological role of CD24 in the previous cancer models illustrated that manipulating CD24 expression level is mirrored by changes in the expression levels of Cten and its downstream targets (FAK and ILK), we sought to detect whether similar modifications in the expression levels of these molecules would be observed in the NSCLC cell lines following manipulation of CD24 expression. An increase in the expression levels of Cten, FAK and ILK were observed because of up-regulation of CD24 in H460 (Figure 4-24, A), while the expression levels of these molecules reduced following down-regulation of CD24 in H226 and A549 cell lines (Figure 4-24, B & C). The changes in the expression levels of EMT markers following CD24 up-regulation H460 were also evaluated. The expression levels of N-cadherin and Snail were seen to increase, while a decrease in the expression level of E-cadherin was observed (Figure 4-25).

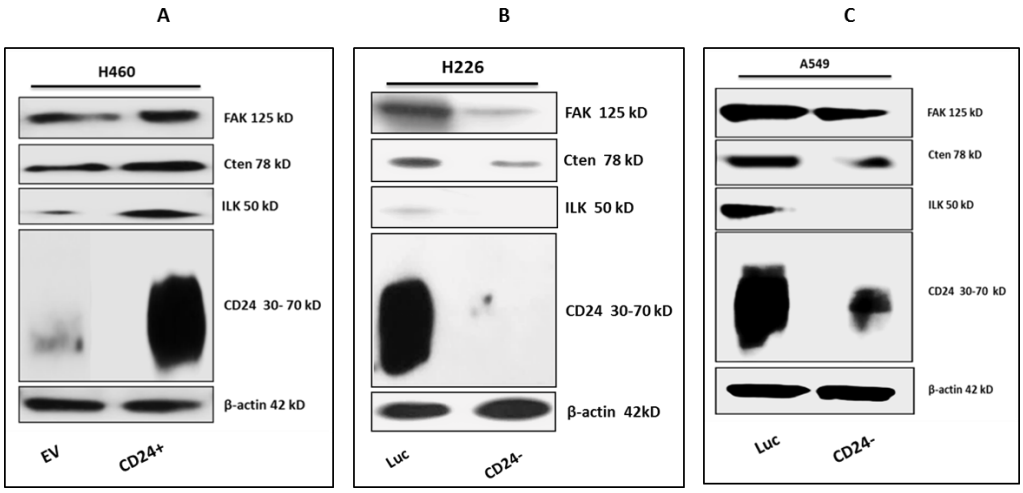


Figure 4-24. Investigation of the CD24 downstream targets in NSCLC cell lines. A dual approach of forced expression of CD24 in a CD24-lower expressor cell line, H460, and knockdown CD24 in two CD24-high expressor NSCLC cell lines, H226 and A549, were performed separately. Western blot data **(A)** demonstrated that CD24 overexpression in the H460 cell line caused an increase in the expression level of Cten, FAK and ILK compared to the EV control. CD24 knockdown in H226 and A549 cell lines led to a decrease in the expression level of Cten, FAK and ILK **(B &C)**. The loading control protein (β-actin) showed the amount of proteins loaded on the gel (25 µg). Transfections and relevant experiments were performed in triplicate.

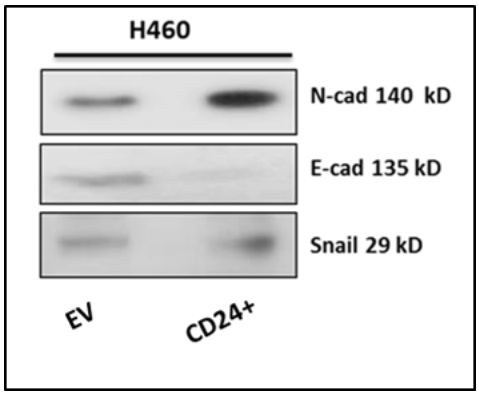


Figure 4-25. Investigation of the relationship between CD24 and EMT markers in NSCLC. CD24 overexpressed in H460 led to an increase in the expression levels of N-cadherin and Snail, while a reduction in the expression level of E-cadherin was observed. The loading control protein (β-actin) showed the amount of proteins loaded on the gel (25 µg). All experiments were performed in triplicate.

4.3 Discussion

In the previous chapter, the potential role of CD24 in the regulation of cell function in four cancer models (CRC, pancreatic, liver, and lung) was investigated. CD24 was found to play a crucial role in regulating several cell functions, including motility, invasion, proliferation, and colony formation in the cell lines of these cancer models. However, it is still uncertain how these functions are regulated by CD24. Therefore, an attempt was made to identify appropriate molecule(s) that could be investigated along with CD24 to study their interaction and/or association in order to determine potential downstream targets and thereby disclose the mechanism of action of CD24 in regulating cellular functions.

The initial focus of study was the tensin family, since it has been identified as regulating various activities that take place at focal adhesions through targeting important integrin signalling molecules such as FAK and ILK (Yam et al., 2009a). Tensin 4 or TNS4 (Cten), a member of the Tensin family, was the ideal molecule to tackle due to its active role in influencing tumour progression as a tumour suppressor, or oncogene, which has been described in several studies (Lo and Lo, 2002, Martuszewska et al., 2009).

Because of its association with integrins in trafficking integrins into lipid rafts, CD24 is more likely to associate with Cten at focal adhesions, as the latter molecule has also been reported to physically interact with integrin cytoplasmic tails (Runz et al., 2008). Moreover, preliminary data have been generated by our group showed that a number of changes induced by modulation of Cten expression in CRC cell lines have also been found to be induced by modulation of CD24 expression (Al-Ghamdi, 2013). Additionally, a phosphokinome array

performed following CD24 down-regulation in CRC cell lines showed an analogous pattern of kinase changes similar to that observed following Cten down-regulation in the same cell lines (AHMED, 2011). These similarities between CD24 and Cten led to the hypothesis that CD24 could be an upstream effector that up-regulates Cten expression. Showing such a relationship between these two molecules may reveal a mechanism by which CD24 controls cell functions and provide a platform for further investigations to determine the downstream targets of both molecules.

In relation to this study, it was hypothesised that certain focal adhesion molecules interact with integrins, and have been reported as downstream targets of Cten such as FAK and ILK, which may also be mediated by CD24 by signalling through Cten. To test this hypothesis, CD24 expression was manipulated either by overexpression in CD24-negative expressor cell lines or knockdown in CD24-positive cell lines of the four cancer models. Changes in the expression of Cten and other focal adhesion molecules, such as FAK, ILK, AKT, and EMT markers including E-cadherin, N-cadherin, and Snail, have been reported to be associated with tumour progression, were evaluated by Western blot and the results confirmed by qPCR.

a) The Mechanistic Basis of CD24-Mediated Induction of Cell Function

i. CD24 is a Positive Regulator of Cten, ILK and FAK

The data presented here showed that the forced expression of CD24 in CD24-negative cell lines of the tested cancer models caused an increased expression of Cten, FAK and ILK. Similarly, knockdown of CD24 in CD24-positive cell lines of the cancer models reduced the expression levels of Cten as well as ILK and

FAK. Together, these results indicate that CD24 is a positive regulator of Cten, ILK and FAK.

Preliminary results from our group indicated that FAK and ILK are downstream targets of Cten (Al-Ghamdi, 2013). It is therefore uncertain whether the effects are independent or whether there is a CD24-Cten-ILK/FAK pathway. Such outcomes may contribute to the identification of a novel mechanism by which cell functions are controlled in these cancer models.

Since the expression levels of FAK and ILK have been shown to be mirrored by the alterations made in the expression of Cten and CD24, this supports the probability of association between CD24 and Cten. Co-transfection of either CD24 or Cten and knockdown of the other gene showed that CD24 is associated with Cten, suggesting that they may collaborate to modulate cell function. The likelihood that these molecules could physically interact with each other was assessed, since both have been reported to be located at focal adhesions and associated with integrins. The data showed that CD24 is immunoprecipitated with Cten, indicating CD24-Cten interaction in a protein complex form.

To confirm whether CD24 regulates FAK and ILK expression by signalling through Cten, the expression levels of these molecules were evaluated in a $SW620^{Cten\ KO}$ cell line, which has been shown to not express Cten following Cten knockout. The data revealed that the knockout of Cten in SW620 cells resulted in a complete disappearance of the expression levels of both FAK and ILK, suggesting that Cten is a positive regulator of FAK and ILK exprerssion, and that CD24 signals through Cten to regulate the expression of FAK and ILK.

The data from the functional assays revealed substantial reductions in cell migration and invasion in $SW620^{CtenKO}$ cells even though CD24 is still present. This finding, together with the previous one, indicates that CD24 up-regulates Cten, FAK and ILK expressions, and regulates cell function through the CD24-Cten-ILK/FAK pathway.

ii. CD24 Stabilizes Cten Protein

The expression of Cten at the mRNA level, evaluated by qPCR following manipulation of CD24 expression, did not change, suggesting that Cten expression is regulated by CD24 at the protein level. It is more probable that this is the result of a mutual protein stabilization rather than mutual gene induction, as CD24 and Cten have been shown to interact. This hypothesis was tested using the CHX assay, which was performed following CD24 forced expression in CD24-negative cell lines or CD24 knockdown in CD24-postive cell lines.

Cells co-transfected with CD24+/Cten+ in CD24-negative cell lines, followed by treatment with CHX, resulted in a noticeable stabilisation of Cten protein. Similarly, cells transfected with a luciferase control in CD24-postive cell lines, followed by treatment with CHX, resulted in the stabilisation of Cten protein. Degradation in the expression level of Cten was detected when CD24 was excluded from the cells. Collectively, these data suggest that CD24 positively up-regulates Cten and that this is mediated through protein stabilisation. This is a novel result that should be considered in the identification of the CD24 signalling pathway through its downstream targets. However, mechanistic insight into Cten up-regulation by CD24 is still lacking.

iii. CD24 Induces EMT

The data presented here showed an association between CD24 and a number of EMT markers. Changes in the expression levels of EMT markers such as N-cadherin, Snail and E-cadherin were evaluated following overexpression and knockdown of CD24 in the four cancer model cells. CD24 was found to up-regulate N-cadherin and Snail, but down-regulate E-cadherin in all the tested cancer model cell lines. Such an association between CD24 and EMT markers is important in the induction of cancer cells, leading to acquisition of cancer invasive and metastatic processes. The EMT markers may be regulated by CD24 through targeting Src, a dependent activation of FAK that has been reported to induce cell activity to promote EMT (Cicchini et al., 2008).

b) The Mechanistic Basis of CD24-Mediated Induction of Stemness

Changes in the expression levels of a number of important proteins, such as β-catenin, Src, and adaptor proteins including Vinculin, Paxillin, were evaluated after CD24 manipulation in different cell lines in an attempt to further identify CD24-associated molecules. The results showed that CD24 positively up-regulates Src expression but inhibits β-catenin expression. It seems that Paxillin and Vinculin expression levels are not involved in the CD24 signalling pathway, as no change was detected in their expression levels following manipulation of CD24 expression. These findings indicate that Src is another downstream target of CD24; however, further investigations to confirm this observation are required. An association between $\alpha_v\beta_3$ integrin and c-Src has been reported, suggesting that Src may play a significant role in the stimulation of anchorage independence and tumour progression (Desgrosellier et al., 2009). Moreover, integrin β3 was shown to interact with c-Src independently of FAK signalling

to enhance stemness and metastasis in pancreatic and breast tumours (Desgrosellier et al., 2014).

i. CD24 Activates the AKT Signalling Pathway

AKT is a target of the PI3K signalling pathway and is associated with cell survival and stemness (Vivanco and Sawyers, 2002). Activation of AKT occurs through independent activation of two phosphorylation sites (Ser473 and Thr308) (Qiao et al., 2008). The phosphorylation of AKT at Thr308 is induced by 3-phosphoinositide-dependent protein kinase-1 (PDK1), which is entirely reliant on PIK3CA (Vanhaesebroeck and Alessi, 2000a), while the phosphorylation of AKT at Ser473 is activated by mTORC2 (Yothaisong et al., 2013, Vivanco and Sawyers, 2002).

In vitro investigations to determine whether CD24 could activate the AKT signalling pathway, thereby stimulating CRC stemness, were conducted in two cell lines, DLD-1 and GP2D, which show constitutive AKT activation. A significant reduction in the expression level of p-AKT (Ser473 – activated by the mutant PIK3CA) was observed following CD24 knockdown in both cell lines, while no significant alteration was detected in the expression level of p-AKT (Thr308). Moreover, CD24 was shown to regulate the activity of AKT downstream targets such as p-eNOS, p-FAK, and p-CREB. It appears that CD24 works specifically on Ser473 and may need priming by the PI3K pathway to phosphorylate Thr308. Together, these results validated preliminary data generated using the phosphokinome array (AHMED, 2011), and supported the possibility that CD24 stimulates stemness through activation of the AKT pathway. Therefore, the Ser473 phosphorylation site of AKT may be a model

marker for AKT activity, and could be a novel marker associated with poor prognosis in CRCs.

ii. Combining PI3K Inhibitor and CD24 siRNA Enhances the Activity of the Drug

It has been suggested that that AKT at Ser473 is a target of CD24 activity, while Thr308 is a target of the PI3K signalling pathway. Therefore, the CD24/PI3K combination was examined. The effect of inhibition of the PI3 kinase pathway with the specific inhibitor LY294002 at concentrations of 10 μM and 50 μM alone or in combination with inhibition of CD24 siRNA in the cell lines GP2D and DLD-1 was evaluated.

Treatments with LY294002 at both the lower and higher dose alone resulted in a reduction in AKT activation at both residues in both cell lines, whilst treatment with CD24 siRNA resulted in a reduction in the AKT activation at the AKT Ser473 residue only. Treatments with CD24 siRNA in a combination with the PI3 kinase inhibitor at both concentrations reduced the activation of AKT at both residues. However, treatment with CD24 siRNA in combination with AKT inhibitor at the lower dose was more effective than the effect of treatment with the higher dose of the AKT inhibitor alone.

Consistent with the effect of these treatments on AKT activation, a combination of these treatments (CD24 siRNA and LY294002 at a lower dose) resulted in significant reductions in both cell motility and invasion equivalent to that observed after treating the cells with LY294002 alone at a higher dose.

Collectively, these results demonstrate that CD24 co-operates with PI3K to activate AKT. The mechanism of the inhibitory effect of CD24-siRNA and PI3 kinase inhibitor LY294002 on AKT activity is unknown; however, combining PI3K inhibitor and CD24 siRNA enhances the activity of the drug, which may be useful in in the development of anticancer drugs.

In summary, these observations indicate that CD24 may directly regulate Cten, probably through integrins, which also regulate expression of FAK and ILK, suggesting that CD24 could form part of a unique mechanism pathway in which cell functions are regulated in the tested cancer models. Furthermore, Cten is stabilized by CD24 and is crucial for mediating the functions of CD24. Seven potential downstream targets of CD24 (i.e., Cten, FAK, ILK, AKT, Src, N-cadherin, and Snail), which may be directly regulated by CD24, or indirectly through other molecules, have been identified. Other possible downstream targets of CD24 need to be studied in order to understand the exact mechanism of CD24 signalling in cancer. Overall, CD24 seems to have features that promote metastasis; however, these *in vitro* data need to be further validated using animal models in future studies.

5 INVESTIGATING THE UPSTREAM REGULATORS OF CD24 IN CANCER

5.1 Introduction

CD24 appears to have a variety of functions, many of which can support development of many different types of tumours including, as we have shown in the previous chapters, tumours of endodermal origin. Despite considerable literature emerging about the significance of CD24 expression in cancer, little is known about the mechanisms by which CD24 regulates cell functions and even less about how CD24 expression is regulated in cancer cells.

Wnt signalling is a well-known oncogenic signalling pathway and the possible association between the Wnt signalling cascade and CD24 expression has been previously studied by our group. Both forced expression of dominant negative TCF-4 (a known mechanism of inhibiting Wnt signalling) and knockdown of ß-catenin (with a specific siRNA) caused a considerable reduction in CD24 expression in the cell lines HT29, GP2D and DLD1. These contain APC mutations and, consequently, have activated Wnt signalling leading to the conclusion that CD24 is a highly possible downstream target of Wnt signalling (Ahmed et al., 2010, AHMED, 2011).

The cytokine interleukin 6 (IL-6)-has a role in the regulation of complex cellular processes, including gene activation, proliferation, and differentiation (Heinrich et al., 1998). Depending on the cell type, IL-6 can act through several standard protein kinase cascades, such as MAPK and PI 3-kinase. Furthermore, IL-6 is able to directly activate STAT factors, such as STAT1 and STAT3, through JAK (Hodge et al., 2005). Since JAK–STAT3 pathway is targeted by IL-6 (Yu et al., 2014), and that the JAK2/STAT3 signalling cascade is essential for growth of $CD44^{+}CD24^{-}$ stem cell-like breast cancer cells (Marotta et al., 2011), this may suggest that the IL6/STAT3 signalling actually is involved in regulating CD24.

Lee et al. reported that CD24 acts as a functional liver-tumour-initiating-cells (T-ICs)-marker that stimulates T-IC genesis via STAT3- mediated NANOG regulation (Lee et al., 2011). Bretz et al. provided evidence that CD24 is involved in the regulation of STAT3 and FAK activities and suggested that Src could be crucial in this process (Bretz et al., 2012b). Together, these findings illustrate the important role of CD24 in regulation of the STAT3 pathway. However, when human dendritic cells (DCs) were stimulated with cytokines and lipopolysaccharide (LPS) in breast cancer cell lines, a reduction in the expression of proto-oncogene HER-2/neu along with CD24 was observed. In addition, inhibition of STAT3 by a specific inhibitor or knocking down of STAT3 with SiRNA in tumour cells resulted in influencing the susceptibility of tumour cells to apoptosis and DC-mediated inhibition of both CD24 and HER-2/neu (Hira and Manna, 2012).

CD24 expression has been reported to be transcriptionally down-regulated by Twist in breast cancer cells and a direct contribution of Twist in producing a breast cancer stem-cell phenotype via down-regulation of CD24 expression was also noticed (Vesuna et al., 2009), this suggests that CD24 does not have cancer inducing properties in breast cancer. In breast cancer also, a further study showed that treating MCF-7, T-47D, and ZR-75-1 cells with a specific estrogen receptor (ER) agonist led to down-regulation of CD24 (Kaipparettu et al., 2008), indicating estrogen-mediated repression of CD24 mRNA. It was also suggested that this repression might occur because of a direct transcriptional effect depending on estrogen receptor alpha (ERα) and histone deacetylases (HDACs). The interaction between ERα and CD24 is ostensibly complex; a preliminary

literature review revealed that down-regulation of CD24 is not always necessarily associated with ERα expression.

CD24 was found to be subject to promoter methylation, and its mRNA expression can originate in CD24-negative breast cancer cell lines by demethylation. Smith et al., found that CD24 may be regulated by Ral GTPases (Smith et al., 2006), and NDRG2, a tumour-suppressor gene, has been described as inhibiting CD24 expression, which sequentially reduced the adhesion and invasion capacities of breast cancer cells (Zheng et al., 2010).

Although significant efforts have been made to identify potential upstream regulators of CD24, the exact mechanism by which cell functions are regulated in tumours remains undetermined.

5.2 Results

Based on the results presented in Chapter 4 that show a close association between CD24 and Cten in different cancer models and the similarities in functional activity between CD24 and Cten, we hypothesised that the already-established upstream regulators of Cten may affect the Cten expression via signalling through CD24. If so, they might be upstream regulators of both molecules and there may be a CD24 to Cten signalling pathway. To address this hypothesis, we investigated likely upstream regulators of CD24 specifically by looking at the effect of KRAS and EGFR (i.e., upstream regulators of Cten) on the CD24-expression level in a number of cancer cell lines.

5.2.1 Validation of the Regulation of Cten Expression by KRAS

Before proceeding to the experimental work, we sought to validate KRAS as an upstream regulator of Cten. This was confirmed by knocking down KRAS in the SW620 cell line (a high-expressor cell line for Cten and which contains mutant KRAS). A Western blot test was then used to evaluate a possible alteration in the expression level of Cten. KRAS knockdown led to a considerable loss in the expression level of Cten (Figure 5-1). This result was consistent with previous data generated by our group (Al-Ghamdi et al., 2011) and suggests that KRAS is a positive upstream regulator of Cten.

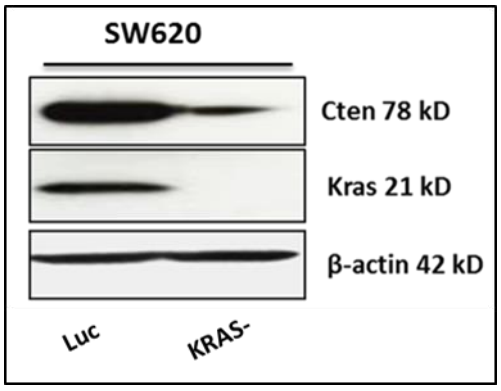


Figure 5-1. Validating the targeting of Cten by KRAS in CRC. KRAS knocked down in SW620 cell line. The Western blotting data showed that knockdown of KRAS resulted in a significant reduction in the expression level of Cten. The loading control (β-actin) showed the amount of protein loaded on the gel (25 µg).

5.2.2 Investigation of the Likelihood of Targeting CD24 by KRAS

Cten is regulated by KRAS in CRC cell lines, as shown above and it has been shown by our group to regulate Cten in pancreatic cancer cell lines (Al-Ghamdi et al., 2011). We therefore sought to investigate whether KRAS was an upstream

regulator of CD24 and whether that would lead to Cten activation. CD24 was knocked down in SW620 and Western blot data illustrated that down-regulation of KRAS led to an increase in the expression level of CD24 (Figure 5-2A). Also, CD24 knockdown led to an increase in KRAS expression (Figure 5-2B). These data were totally unexpected and suggest that KRAS in an inhibitor of CD24. In addition, CD24 appears to be an inhibitor of KRAS. Furthermore, both proteins are positive regulators of Cten and, as shown in the previous chapter, CD24 and Cten are mutually stabilizing although this effect is much less than the direct effect of KRAS activity. Taken together, these findings suggest that CD24 does not integrate with the KRAS signalling pathway in terms of regulating Cten. Since the KRAS oncogene has been found to activate multiple signalling pathways, KRAS may regulate Cten via a signalling pathway other than CD24.

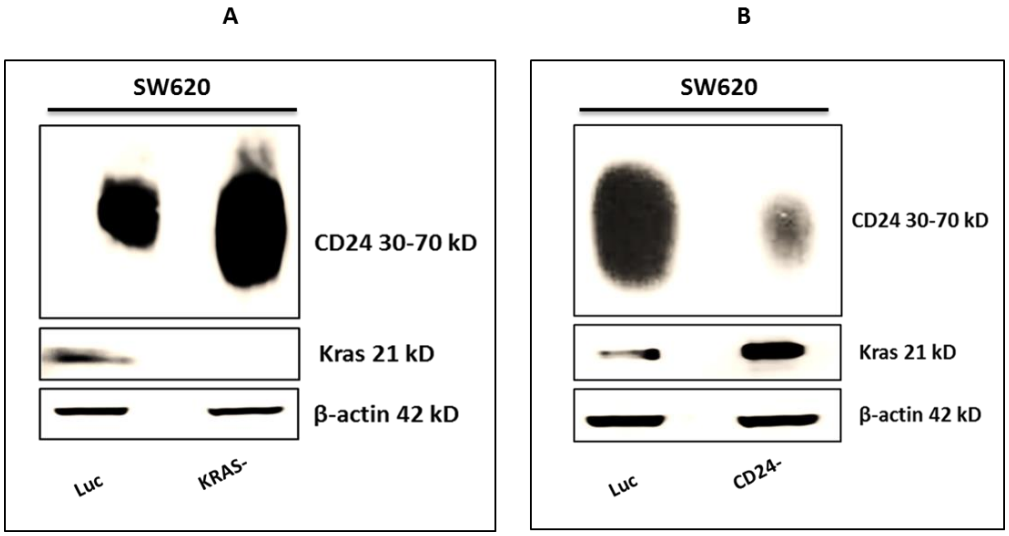


Figure 5-2. Investigating the regulation of CD24 expression by KRAS in CRC. Both KRAS and CD24 were knocked down in the SW620 cell line. Immunoblotting data showed that down-regulation of KRAS led to an increase in the CD24 expression level **(A)**. Similarly, CD24 knockdown in the same cell line resulted in an increase in KRAS expression **(B)**. The loading control (β-actin) showed the amount of protein loaded on the gel (25 µg). All experiments were performed in triplicate.

5.2.3 Investigation of the Likelihood of Targeting CD24 by the Epidermal Growth Factor Receptor (EGFR)

Another potential upstream regulator of CD24 was the EGF/EGFR signalling pathway – known to be a positive regulator of Cten and KRAS. To assess whether CD24 may be involved in the EGFR signalling pathway mechanism, the experimental work had to be conducted in cell lines that are wild-type for KRAS. EGFR was inhibited in the CRC cell line HT29 (a wild-type for KRAS (Fadhil et al., 2010), and in the NSCLC cell line H226 (a wild-type for KRAS (Franklin et al., 2010) using the commercially available EGFR inhibitor PD135053. Cells were incubated with PD135053 for 24 hrs at a concentration of 20 µM as detailed in Chapter 2, followed by Western blotting.

Following the inhibition of EGFR in HT29 cell line, a significant reduction in the expression levels of KRAS, CD24 and Cten were observed (Figure 5-3). There was a reduction in the expression level of total EGFR, but greater reduction was observed in the expression level of phosphorylated-EGFR (p-EGFR, the activated form of the EGFR). This suggests that inhibition of EGFR activity could reduce stability of EGFR protein). These observations were replicated when EGFR protein activity was inhibited in H226 cells.

To further investigate the regulation of CD24 by the EGFR signalling pathway, the CD24 mRNA was evaluated using qPCR after treating HT29 and H226 cells with EGFR inhibitor PD135053 at 20 µM. qPCR data demonstrated significant changes in the CD24 mRNA expression in both cell lines ($p=0.0209$ and $p=0.0133$, respectively) compared to the negative controls (Figure 5-4).

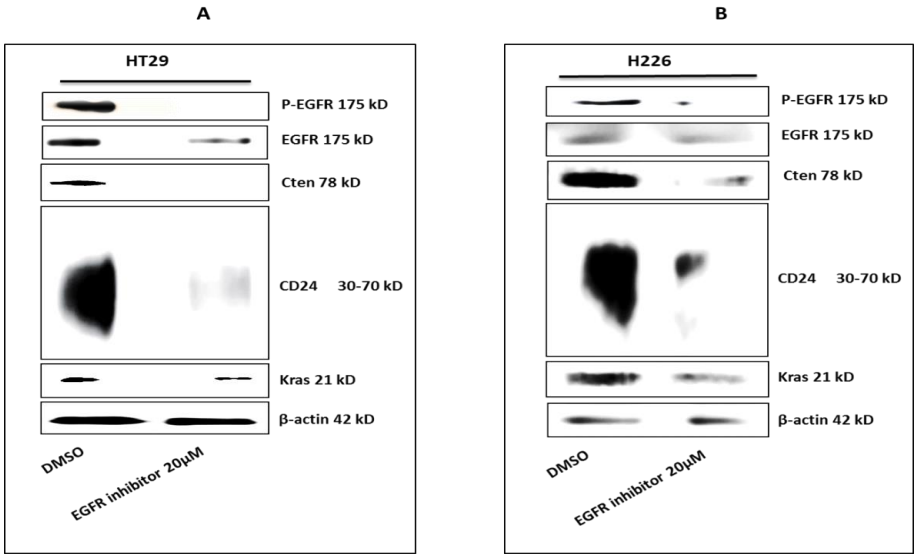


Figure 5-3. Investigating the regulation of CD24 by EGFR in cancer cell lines. EGFR inhibited in HT29 **(A)** and H226 **(B)** cells when used at a 20 μM concentration. Western blotting data showed that inhibiting EGFR in both cell lines resulted in a high reduction in the expression level of KRAS, CD24 and Cten. We also noticed that EGFR inhibition in both cell lines caused a significant depletion in p-EGFR expression compared to EGFR expression. The loading control (β-actin) showed the amount of protein loaded on the gel (25 μg). All the experiments were repeated in triplicate.

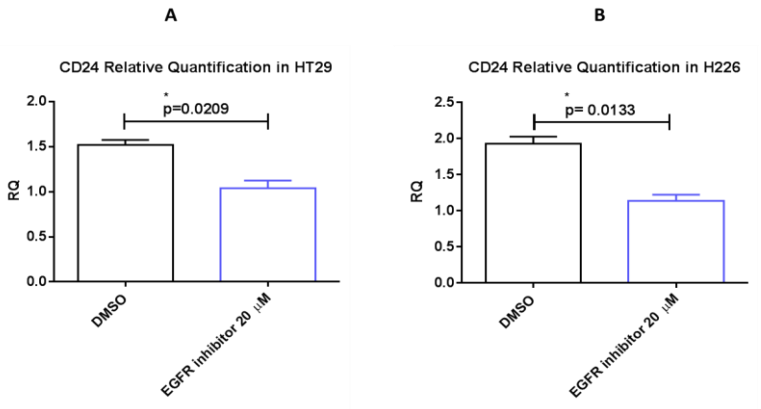


Figure 5-4. CD24 relative quantification after inhibiting EGFR in cancer cell lines. CD24 mRNA levels in RNA samples were evaluated following EGFR inhibition in HT29 **(A)** and H226 **(B).** A significant reduction in the CD24 mRNA levels were observed in both cell lines (p=0.0209 and p=0.0133, respectively) compared to the control DMSO control. The quantity of Cten mRNA in each cell line was normalised to the expression of housekeeping gene HPRT1 in each cell line. Each experiment was conducted in triplicate, and the data was analysed using Prism software (unpaired t-test).

In order to further confirm whether the EGFR signalling pathway is involved in regulating CD24 in cancer, we stimulated RKO cells that express EGFR at very low levels and, in the meantime, is a wild-type for KRAS, with recombinant EGF PHG0333L (20 ng/ml) and with DMSO as a control. Such stimulation resulted in an increase in the expression levels of KRAS, CD24 and Cten (Figure 5-5).

Together, these results suggest that EGFR may regulate CD24 gene expression. We have previously shown that EGF signalling results in increase in Cten protein levels but no increase in Cten mRNA expression (Thorpe et al., 2015), and thus we can hypothesize that Cten is a downstream target of EGFR signalling through stabilization by CD24. The role of KRAS is uncertain since, in the context of EGFR signalling, it would appear to be a positive regulator of CD24.

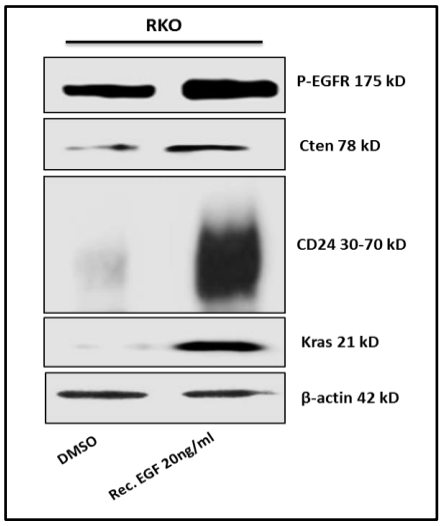


Figure 5-5. Validating the relationship between EGFR and CD24. RKO cells were stimulated with recombinant-EGF at a 20ng/ml concentration. A significant increase in the expression level of KRAS, CD24 and Cten was observed following EGF stimulation. The loading control (β-actin) showed the amount of protein loaded on the gel (25 µg).

5.2.4 Investigating the Effect of EGFR on Cell Motility in CRC

Many signalling pathways were found to be activated by an EGFR signalling cascade in response to EGF stimulation (Andl et al., 2004, Oda et al., 2005). One of these signalling events includes the tyrosine phosphorylation of signal transducer STAT3, while another includes stimulation of the phosphatidylinositol 3-OH kinase pathway.

Our data showed that CD24 appears to be regulated by EGFR signalling and, in turn it regulates Cten. Since both CD24 and Cten are able to induce cell motility, we investigated whether EGFR signalling was involved in cell motility. To examine this hypothesis, the Transwell migration assay was performed following (a) the inhibition of EGFR in HT29 and H226 using an EGFR inhibitor at a 20 μM concentration and (b) the stimulation of EGF in RKO cells with recombinant-EGF at a 20ng/ml concentration. It was observed that cell motility was reduced significantly after inhibiting EGFR in the HT29 and H226 cells ($p=0.0002$ *and* $p<0.0001$; Figure 5-6A & B). Likewise, stimulating EGF in RKO cells resulted in a substantial increase in cell motility ($p<0.0064$, Figure 5-6 C). Together, these findings match those presented in Chapter 3 following the knockdown of CD24 in the same cell lines, suggest a possible functional relationship between EGFR signalling and CD24 in terms of regulating cell behaviour.

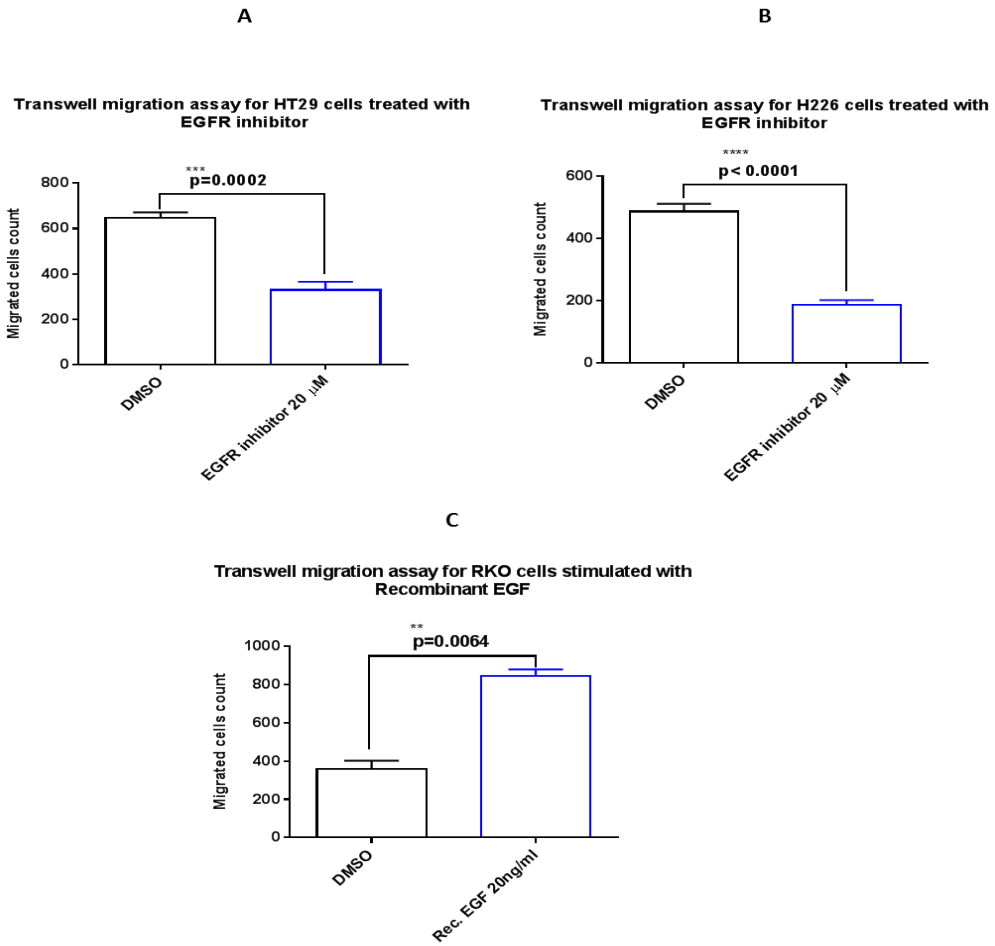


Figure 5-6. The effect of EGFR on cell motility. EGFR inhibited in HT29 and H226 using EGFR inhibitor at a 20 µM concentration, while it stimulated in RKO cells using recombinant-EGF at a 20ng/ml concentration. Inhibiting EGFR significantly reduced cell motility in HT29 and H226 ($p<0.05$; **A** and **B**). Stimulating EGF in RKO cells caused a significant increase in cell motility ($p<0.05$, **C**). The assays were performed in triplicate, and the data were analysed using Prism Pad 6 software (unpaired t-test).

5.3 Discussion

In the previous chapters, we illustrated that CD24 up-regulates Cten in different cancer models cell lines. In addition, Cten is stabilized by CD24 and is essential for mediating the functions of CD24. Our group has previously shown that Cten is up-regulated by EGFR and KRAS signalling (Al-Ghamdi et al., 2011) and Katz et al. found Cten to be up-regulated by EGFR signalling in breast cancer (Katz et al., 2007). KRAS is a downstream target in EGFR signalling and therefore, taking these data into account, we hypothesised that EGFR/KRAS signalling positively regulates CD24 signalling and CD24 in turn positively regulates Cten.

Before going further, we wanted to again validate that KRAS is a positive regulator of Cten. Therefore, KRAS was knocked down in the SW620 cell line using a KRAS-specific siRNA and, as expected, there was a significant reduction in Cten expression. We next evaluated CD24 expression following KRAS knockdown. Unexpectedly, KRAS knockdown in SW620 resulted in an increase in CD24 expression suggesting that KRAS may inhibit CD24. Given this observation, we tested KRAS expression following CD24 knockdown and we noticed that CD24 knockdown in SW620 led to an increase in KRAS expression.

Together, our data support the existence of a mutually inhibitory relationship between KRAS and CD24. This was made even more interesting given the fact that KRAS unequivocally positively regulates Cten and, since Cten stabilizes CD24, knockdown of KRAS would be expected to cause a reduction in Cten and a consequent reduction in CD24. KRAS is at the centre of a number of signalling pathways and our results suggest that some of these may be inhibitors of CD24

and the effect of the inhibition is greater than the effect of CD24 stabilization by Cten.

Next, we turned to investigating the other hypothesis that suggested EGFR as a potential upstream regulator of CD24. Despite the data from the KRAS knockdown experiments, we felt it was worthwhile to continue with this experiment. Although KRAS is a crucial part of the EGFR signalling pathway, we have found that the activity of KRAS when activated by EGFR signalling may be different to the activity observed when it is directly targeted. Our previous work on the relationship between Cten and KRAS had shown that EGFR signalling (through KRAS) led to up-regulation of Cten through protein stabilization without change in Cten mRNA; in contrast, knockdown of KRAS led to down-regulation of Cten through inhibition of Cten gene expression causing a reduction in Cten mRNA (Thorpe et al., 2015).

We investigated whether EGFR signalling could influence CD24 expression. A dual approach was used i.e. EGFR was inhibited with PD135053, a specific EGFR tyrosine kinase inhibitor and EGFR was activated by stimulating with EGF. The experimental work was conducted in cell lines from CRC and NSCLC, and these were wild-type for KRAS (Fadhil et al., 2010, Franklin et al., 2010). The objective of selecting cell lines without KRAS mutations for the investigations was to avoid any potential confounding effect of constitutive KRAS activation due to mutation. The data showed that EGFR signalling is a positive regulator of KRAS, CD24 and Cten leading to increased levels of protein. Analysis of the mRNA showed that there was induction of the CD24 gene although the increase in Cten was probably due to protein stabilization by CD24. Functional analysis showed that EGFR signalling did induce motility and

that it could be through the CD24-Cten activation. The role of KRAS is uncertain. Our data from the KRAS knockdown would suggest that KRAS in an inhibitor of CD24 and thus it is possible that EGFR signals to CD24 through an alternative pathway (such as PI3K) whose effect is greater than that of the induced KRAS. Alternatively, it could be that context matters and the activity of KRAS in the EGFR pathway may be different to the activity observed following direct knockdown of KRAS.

To our knowledge, this is the first time that EGFR has been suggested as upstream regulator of CD24. It is not altogether surprising as an abundant body of literature illustrates that both EGFR and CD24 play a significant role in the regulation of cell functions and have some common downstream targets. For instance, EGFR has been found to regulate cell motility by affecting FAK expression that was assumed to be regulated through other pathways. We also established FAK as a downstream target of CD24, as was shown in Chapter 4.

To sum up, we have succeeded in identifying a new potential upstream regulator of CD24 (i.e., EGFR) to create an EGFR-CD24-Cten signalling pathway. However, the role of KRAS in this pathway is uncertain.

6 INVESTIGATING THE SUBCELLULAR LOCALISATION OF CD24

6.1 Introduction

CD24 has been reported to be involved in tumour progression and metastasis of several solid tumours, including colorectal, ovarian, breast, prostate, gastric, pancreatic and non–small–cell lung cancer (Pearson et al., Kristiansen et al., 2003a, Jacob et al., 2004). In lung cancer, CD24 expression was immunohistochemically examined in NSCLC tissue, and CD24 expression was found to be associated with adenocarcinoma-type histology, advanced stage disease and cancer-related death (Lee et al., 2010).

An immunohistochemical study on colorectal cancer conducted by Weichert et al. (Weichert et al., 2005a) suggested that the cytoplasmic CD24-staining pattern is prognostically more informative than membranous staining in CRC. However, the biological importance of cytoplasmic CD24 expression is still indeterminate. Choi et al. (Choi et al., 2005), found that CD24 is a likely prognostic marker in epithelial ovarian cancer. This study investigated a larger cohort of borderline ovarian tumours; this provided researchers with an opportunity to link cytoplasmic CD24 immunoreactivity to microinvasion. Ostensibly, shifting from apical membranous CD24 localisation to cytoplasmic CD24 localisation mirrors the transition of epithelial cells to a further invasive phenotype. This is suggestive of the well-known EMT of cancer cells, which is associated with the onset of invasiveness (Weichert et al., 2005a).

Our research group (Ahmed et al., 2009a) investigated the prognostic value of CD24 expression by immunohistochemistry in 345 CRC on a tissue microarrays (TMAs). No association between CD24 expression and poor prognosis was found. This study did however, find a variety of subcellular locations of CD24. In agreement with other studies, CD24 expression was detected on the cell

luminal surface and in the cytoplasm. In addition, the study found that there was expression of CD24 in the nucleus. Given that CD24 is a GPI anchored molecule, its localisation to the nucleus would appear to be a complicated process. However, IHC is known to be a test which can generate reproducible artefacts due to binding to other epitopes / molecules. We were therefore prompted to wonder whether CD24 localisation to the nucleus was a true phenomenon.

The aforementioned data inspired us to investigate the subcellular localisation of CD24. In addition, we sought to investigate whether the expression of CD24 was associated with expression of its proposed downstream targets. This was tested in a series of 84 CRCs and 58 NSCLCs arrayed onto TMAs.

6.2 Results

6.2.1 Investigation of Cytoplasmic and Nuclear Localisation of CD24 in Cancer Cell Lines

The main objective of this study was to further evaluate CD24 localisation in the subcellular components, particularly in the nucleus, as few studies have been conducted in relation to this. Lysates from the SW620 CRC cell line and the A549 lung cancer cell line were fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (as detailed in Chapter 2). This procedure typically permits stepwise separation and extraction of a variety of cellular components such as cytoplasm, membrane, and nuclear soluble proteins, in addition to extraction of cytoskeletal proteins.

We also knocked down CD24 in these cell lines in order to ascertain whether there was any major difference in stability between the CD24 in these

compartments. CD24 expression (cytoplasmic or nuclear) was evaluated using Western blot. In both cell lines, CD24 expression was clearly observed in the cytoplasm as well as in the nucleus. The CD24 cytoplasmic expression was greater than nuclear expression (Figure 6-1). The anti-tubulin and anti-lamin antibodies were used along with anti–CD24 antibody. The first antibody detects cytoplasmic expression only, while the second detects nuclear expression and these were highly expressed in the appropriate compartment indicating there was little contamination of the cytoplasmic fraction with nuclear proteins and there was little contamination of the nuclear fraction with the cytoplasmic fraction. This suggested that the CD24 expression reflected by anti-CD24 in both the cytoplasm and nucleus were accurate. The knockdown experiments showed no differences in stability between the cytoplasmic and nuclear components. This observation, confirms CD24 localisation to the cytoplasm and into the nucleus.

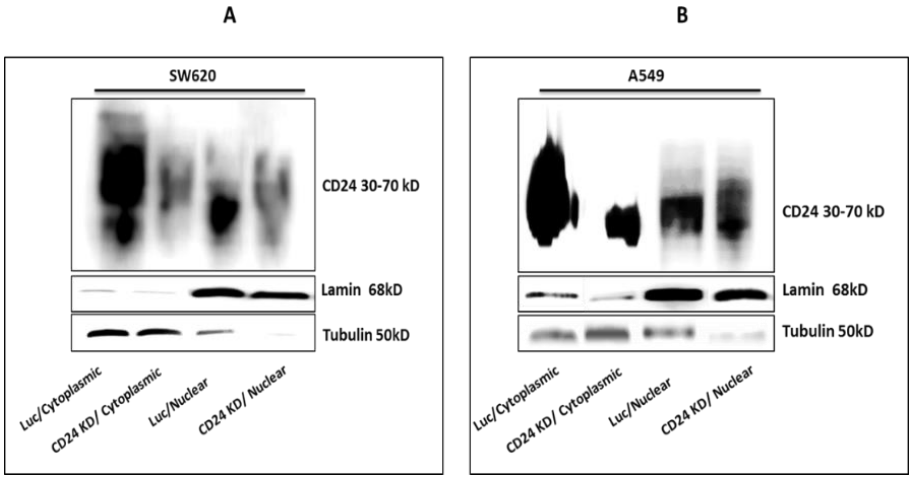


Figure 6-1. CD24 cytoplasmic and nuclear expression in colorectal and lung cell lines. CD24 was knocked down in SW620 and A549 cell lines, followed by a protein fractionation procedure. Western blot was then used to evaluate the cytoplasmic and nuclear CD24 expression. Anti-lamin and anti-tubulin antibodies were included as controls for nuclear and cytoplasmic expression indicators, respectively. In both cell lines, we observed that CD24 is heavily expressed in the cytoplasm and, to a lesser extent, in the nucleus. The loading control showed the amount of proteins loaded on the gel (25 µg).

6.2.2 Investigation of Subcellular Localisation of CD24 and its Associated Molecules in Colorectal Cancer Tissue

The study's purpose was to determine whether CD24 expression in human tissues was correlated with expression of its downstream targets including Cten, FAK, ILK, AKT, Snail, and Src. A total of 84 CRCs were arrayed on a TMA and these were tested by IHC. Features of CRCs TMA cases were all adenocarcinoma. The TMA was constructed by Dr Saira Sajid and Dr Maham Akhlaq, and consisted of cores taken from the central region, luminal surface, and edges of the CRC tumour and from the matched non-tumour (normal) colonic tissue from each case.

For the immunoreactivity assessment, immunohistochemical scoring (H-score) was determined by calculating the percentage of positively stained tumour cells (minimum was 0 and maximum was 100%) which fell into one of three staining categories of 1+ (weak intensity of staining), 2+ (moderate intensity of staining) or 3+ (strong staining). The formula for calculating the H-score was as follows: H-score = [1 x (% cells 1+) + 2 x (% cells 2+) + 3 x (% cells 3+)]. IHC staining was considered positive when at least 10% of tumour cells showed positive staining (at any intensity). Tumours with staining in less than 10% staining were considered negative. The H-score range was 0–300. The expression of each marker was evaluated individually and then correlated to clinicopathological tumour features when possible. Immunostaining of each marker was independently evaluated and checked by a clinical pathologist colleague.

6.2.2.1 CD24 Immunostaining in CRC Tissue

The staining intensity of CD24 in CRC tissue and non-tumour (adjacent to the tumour) colonic tissue showed that the subcellular localisation of CD24 was detected by monoclonal antibody SWA11 (Figure 6-2). In tumour cases, CD24 was predominantly (90%) localised to the cytoplasm, while its nuclear localisation was detected in 30/84 (35.7%) cases. Membranous CD24 immunoreactivity was also seen in 6/84 (7%) tumour cases (Figures 6-2 and 6-3). The frequency of CD24 expression in a variety of CRC tissue sites and matched normal colon tissue was investigated. The highest H-score for CD24 immunoreactivity staining in CRC tissue sites was 300, and the lowest was 30. In normal colon tissue, 100 was highest and 0 was lowest.

We also noticed that there was a significant difference in CD24 expression between tumour and non-tumour (normal) colonic tissues (p=0.017). In most cases, CD24 staining in each cellular component was not associated with tumour grade, tumour stage, nodal stage, vascular invasion, Dukes' stage, or resection margin. However; in some cases, a low CD24-nuclear expression was associated with tumour stage and nodal stage (Table 6-1).

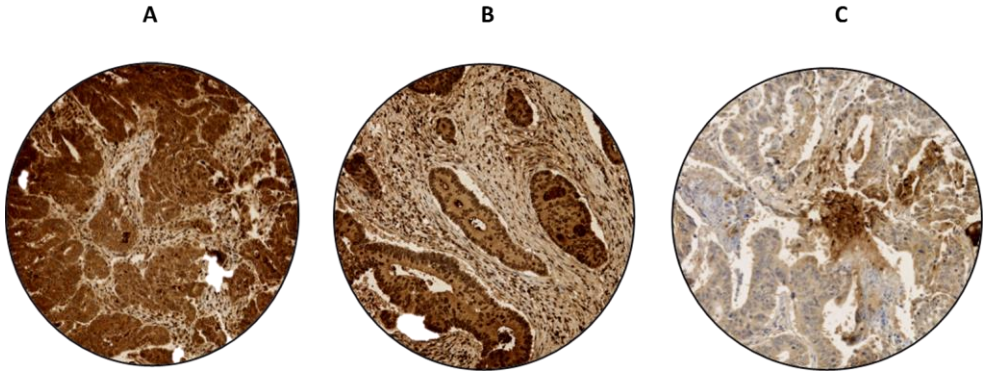


Figure 6-2. CD24 staining intensity in CRC tissue. (A) High (strong) CD24 expression. **(B)** Moderate CD24 expression. (**C)** Low (weak) CD24 expression in the tumour and matched non-tumour tissues. *The pictures were acquired at 5x.*

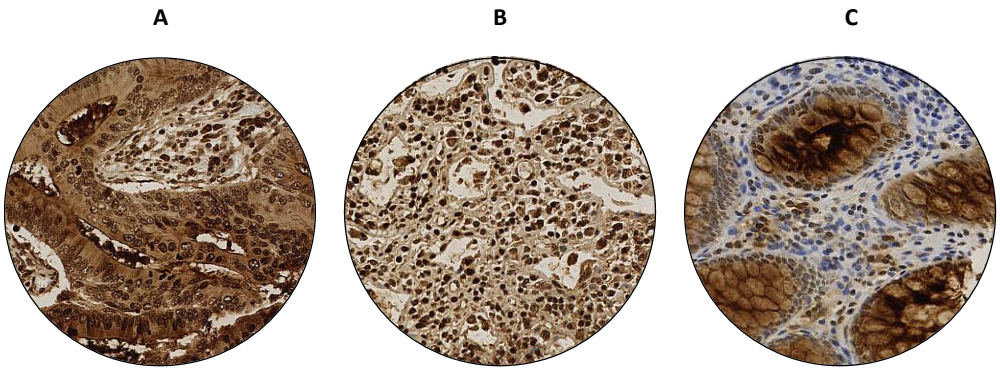


Figure 6-3. Subcellular CD24 expression in CRC tissue. **(A)** CD24 cytoplasmic expression. **(B)** Nuclear expression. **(C)** Membranous expression. *The pictures were acquired at 10x.*

Table 6-1. The correlation between CD24 expression and the clinicopathological features in CRC tumour tissue.

	Cytoplasmic Staining			**Nuclear Staining**		
Parameter	**Low/ Moderate**	**High**	p-value	**Low/ Moderate**	**High**	p-value
Tumour Grade						
1	0	2	0.25	1	1	0.45
2	35	42		51	26	
3	1	4		2	3	
Tumour Stage						****
1	1	2		0	3	**< 0.0001**
2	4	8	0.46	7	5	
3	18	28		40	6	
4	13	10		7	16	
Nodal Stage						*
0	24	33	0.81	39	13	**0.014**
1	10	11		12	12	
2	2	4		2	5	
Vascular Invasion						
0	20	22		30	12	0.38
1	16	24	0.36	23	17	
2	0	2		1	1	
Dukes' Stage						
A	4	9	0.15	6	7	0.51
B	20	18		26	12	
C1	12	17		18	10	
C2	0	4		3	1	
Resection margin						
	35	46	1.00	49	30	0.29
	1	2		3	0	

6.2.2.2 Cten Immunostaining in CRC Tissue

The subcellular localisation of Cten (i.e., a downstream target of CD24) was also investigated in CRC tissue. Like CD24, Cten was found mostly localised to the cytoplasm in the majority of tumour cases, while Cten nuclear localisation was observed only in 4/84 (4.8%) of the total cases. Surprisingly, membranous Cten positive staining was also detected in 20/84 (23.8%) of total cases. We did not detect any significant difference in Cten expression amongst tumour and normal colon tissue ($p=0.37$). Though 90% of normal colon tissue exhibited positive Cten staining, it was more intense in the tumour specimens. In addition, immunoreactivity staining showed that high Cten expression was correlated with 75% of CD24 high-intensity staining.

6.2.2.3 FAK Immunostaining in CRC Tissue

In tumour tissue, cytoplasmic expression of FAK was shown in most CRCs cases, while only 6/84 (7%) cases were positive for FAK nuclear expression. Overall, a total of 33/84 cases (39%) showed high FAK expression, whereas 30/84 (36%) presented low FAK expression. In association with CD24, immunoreactivity staining showed that high FAK expression was correlated with 69% of cases with high intensity CD24 staining.

6.2.2.4 ILK Immunostaining in CRC Tissue

Cytoplasmic ILK expression was apparent in most tumour cases, while ILK nuclear expression was also seen in 4/84 (5%) of cases. High ILK expression was detected in 30/84 cases (36%), while low ILK expression was seen in 33/84 (39%) cases. Immunoreactivity staining revealed that high ILK expression was correlated with 63% of CD24 high-intensity staining cases.

6.2.2.5 AKT Immunostaining in CRC Tissue

Cytoplasmic and nuclear expressions of pan-AKT, as well as phosphorylated AKT (p-AKT) at the Thr-308 site, were studied in CRC tumour cases. Of the 84 cases, 20 (23.8%) showed high pan-AKT expression, while 53/84 (63%) showed low pan-AKT expression. Immunoreactivity staining revealed that high pan-AKT expression was correlated with 42% of CD24 high-intensity staining cases.

Regarding the expression of p-AKT at both sites (Thr-308 and Ser473), high expression of p-AKT (Thr-308) was observed in 27/84 (32%) cases, while 40/84 cases (48%) displayed low p-AKT Thr-308 expression. In association with CD24, immunoreactivity staining showed that high p-AKT (Thr-308) expression was correlated with 56% of CD24 high-intensity staining cases.

6.2.2.6 Snail Immunostaining in CRC Tissue

Snail expression was detected in a high proportion (80%) of tumour cases, and the staining intensity was frequently granular in appearance. High Snail expression was seen in 23/84 (39%) cases, while 41/84 (49%) cases displayed low Snail expression. In terms of correlated immunoreactivity staining, high Snail expression was correlated with 48% of CD24 high-intensity staining cases.

6.2.2.7 Src Immunostaining in CRC Tissue

Cytoplasmic and membranous expression of Src was detected in the vast majority (85%) of tumour cases showing positive staining. Its nuclear expression was also detected in 8/84 (10%) tumour cases. A total of 28/84 (33%) cases showed high Src expression, and 43/84 (51%) showed low Src expression. In addition, immunoreactivity staining showed that high Src expression was correlated with 58% of CD24 high-intensity staining specimens. The overall

correlation between CD24 and its associated molecular partners in CRC tissue is summarised in Figure 6-4 and Tables (6-2 and 6-3).

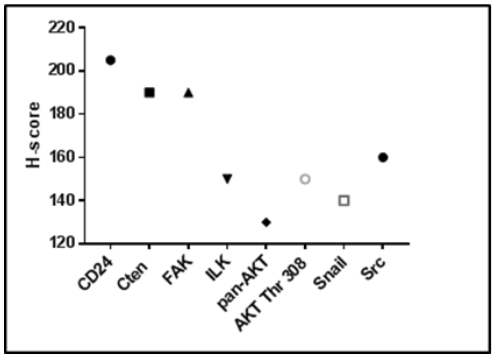
Table 6-2. The correlation between the expression of CD24 and its associated molecular partners in CRC tissue.

Marker	High expression	Moderate	Low expression	*p*- value
CD24	48	11	25	--
Cten	36	28	20	0.008 **
FAK	33	21	30	0.042*
ILK	30	21	33	0.015 *
Pan-AKT	20	11	53	<0.0001 ****
AKT Thr 308	27	17	40	0.005 **
Snail	23	20	41	0.0005 ***
Src	28	13	43	0.006 **

Table 6-3. Median H-score for tested markers in CRC tissue.

Marker	CD24	Cten	FAK	ILK	Pan-AKT	p-AKT Thr 308	Snail	Src
Median H-score	205	190	190	150	130	150	140	160

A



B

Marker	*p*-value
Cten	0.051
FAK	0.051
ILK	0.004**
pan-AKT	0.002**
AKT Thr 308	0.004**
Snail	0.003 **
Src	0.006**

Figure 6-4. The association between CD24 and its downstream targets in CRC tissue based on the median H-score. Significant p-value represents a low association.

6.2.3 Investigation of the Subcellular Localisation of CD24 and its Associated Molecules in Lung Cancer Tissue

The localisation of CD24 in addition to associated molecules mentioned in the previous section were also studied immunohistochemically in a total of 58 NSCLC cases on TMA. Attributes of NSCLC TMA cases were adenocarcinoma (n=49), carcinoid (n=6) and squamous cell carcinoma (n=3). The TMA was constructed by Dr Wakkas Fadhil and consisted of cores taken from different areas of tumours and matched non-tumour lung tissues.

In this portion of the study, we aimed to determine the frequency of CD24 expression and that of its associated molecules in NSCLC. The same systematic approach used in the assessment of immunoreactive intensity in CRC tissue was applied. Following the staining, digital images acquired by the NanoZoomer slide scanning system were used for independent immunoreactivity assessment of each marker. The expression of CD24-associated markers was then correlated to CD24 expression.

6.2.3.1 CD24 Immunostaining in NSCLC Tissue

The staining intensity of CD24 in NSCLC tissue was detected by CD24's monoclonal antibody, clone SWA11 (Figure 6-5). CD24 was detected in multiple subcellular compartments, including the cytoplasm, nucleus, and membrane (Figure 6-6). A high level of CD24 expression was observed in the cytoplasm in 32/58 (55%) cases compared to nuclear expression, was which detected only in 17/58 (29%) cases. Membranous CD24 expression was also observed in 3/58 (7%) of cases. The highest median H-score for CD24 staining intensity was detected amongst carcinoid cases (167) followed by ADC cases (137) and, lastly, in SCC cases (122). Overall, the highest H-score for CD24

immunoreactivity staining in NSCLC tissue was 300 and the lowest was 0, while in non-tumour lung tissue, 200 was highest and 0 was lowest.

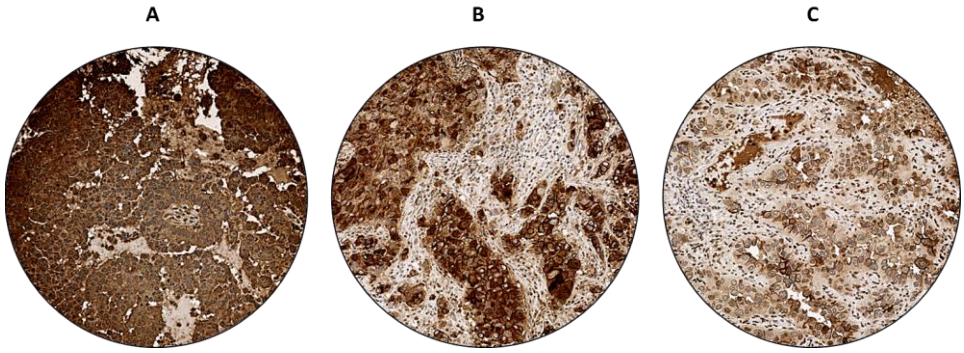


Figure 6-5. The staining intensity of CD24 in NSCLC tissue. (A) High (strong). (B) Moderate. (C) Low (weak). *The pictures were acquired at 5x.*

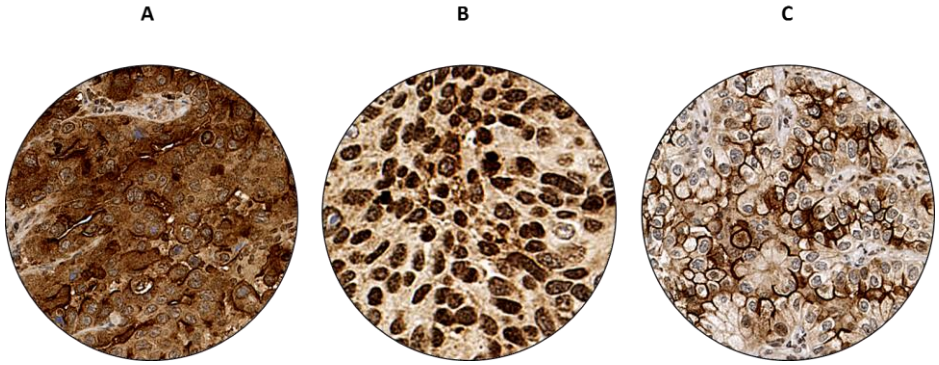


Figure 6-6. Subcellular expression of CD24 in NSCLC tissue. (A) Cytoplasmic expression. (B) Nuclear expression. (C) Membranous expression. *The pictures were acquired at 10x.*

6.2.3.2 Expression of CD24-Associated Molecules in NSCLC Tissue

The staining intensity of CD24-associated molecules (i.e., downstream targets), including Cten, FAK, ILK, AKT, Snail, and Src, were also studied in NSCLC tissue. The aim of this portion of the study was to determine whether there was an association between the expression of CD24 and the above-mentioned molecules and whether it was associated with cancer features such as metastasis. Therefore, data analysis of immunostaining of each marker was performed

identically to that of CD24 staining to determine H-score, staining intensity, and the association between these markers and CD24 expression. Correlations between CD24 and expression of its associated molecules are summarised in Figure 6-7 and Tables (6-4 and 6-5).

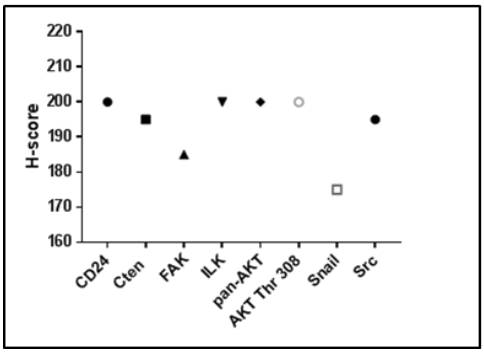
Table 6-4. The correlation between the expression of CD24 and its associated molecular partners in NSCLC tissue.

	High expression	Moderate expression	Low expression	*p*-value
CD24	32	20	6	--
Cten	26	31	1	0.04*
FAK	30	18	8	0.81
ILK	30	26	2	0.24
Pan-AKT	28	28	2	0.17
p-AKT Thr 308	29	29	0	0.02*
Snail	20	31	12	0.03*
Src	31	23	3	0.54

Table 6-5. Median H-score for tested markers in NSCLC tissue.

Marker	CD24	Cten	FAK	ILK	Pan-AKT	p-AKT Thr 308	Snail	Src
Median H-score	200	195	185	200	200	200	175	195

A



B

Marker	*p*-value
Cten	0.2929
FAK	0.0513
ILK	>0.99
pan-AKT	>0.99
AKT Thr 308	>0.99
Snail	0.0194*
Src	0.2929

Figure 6-7. The association between CD24 and its downstream targets in NSCLC tissue considering the median H-score. Significant p-value represents a low association.

6.3 Discussion

a) Subcellular Localisation of CD24 in Tumour Cell Lines

In this study, the subcellular localisation of CD24 in cancer cells was assessed, as a considerable amount of literature has shown that CD24 localises to the cytoplasm; however, few studies have shown its nuclear expression. CD24 localisation in these subcellular cancer components was investigated in appropriate cell lines using Western blotting following protein fractionation. Anti-tubulin antibody can be used to detect cytoplasmic expression and anti-lamin antibody can be used to detect nuclear expression. These control antibodies were used to ensure that fractionation was robust and that the results were not artefacts of contamination during the protein fractionation procedure. Our findings were consistent with previously published data that showed that CD24 can localise to both the cytoplasm and nucleus in cell lines from CRC and NSCLC (Weichert et al., 2005a, Kristiansen et al., 2003a, Yang et al., 2014).

In tumour tissues, the expression of CD24 was immunohistochemically studied in 84 CRC and 58 NSCLC cases. In both CRC and NSCLC, CD24 was found to be overexpressed in both the cytoplasm and the nucleus. In CRC, cytoplasmic expression was detected in 76/84 (90%) cases, compared to nuclear expression, which was detected in 30/84 (36%) cases. In NSCLC, CD24 cytoplasmic expression was seen in 32/58 (55%) cases, while nuclear expression was seen in 17/58 (29%) cases. While CD24 in the nucleus in CRC has been previously described (Ahmed et al., 2009b), this is the first demonstration of nuclear CD24 in NSCLC. The demonstration of nuclear CD24 expression in two different cancer models using two different methods suggests that localisation is a true phenomenon. Furthermore, the knockdown of CD24 using siRNAs was

followed by a reduction of CD24 in both compartments, thereby further supporting the concept of nuclear localisation. This experiment also showed that there was no overt difference in stability between cytoplasmic and nuclear CD24.

High CD24 expression in the cytoplasm has been previously observed and associated with shortened CRC (Weichert et al., 2005a) and NSCLC (Kristiansen et al., 2003a) patient survival. The functional significance of CD24 nuclear expression is uncertain as is the mechanism by which it localises to the nucleus. It is a small but heavy glycosylated protein, which may allow it to interact with other proteins in the nucleus.

With regard to staining intensity, CD24 cytoplasmic staining was of generally heterogeneous intensity. However, nuclear staining was found to be of high intensity. Membranous expression of CD24 was surprisingly infrequently detected. Generally, these results are in line with the findings of previous studies (Wang et al., 2010, Ahmed et al., 2009a, Jaggupilli and Elkord, 2012).

Regarding clinicopathological features, CD24 expression in subcellular components was generally not associated with tumour grade, staging, vascular invasion or resection margin, except in a few cases where CD24 nuclear overexpression was significantly correlated with the presence of lymph-node metastasis and more advanced pathological stage.

b) Association of CD24 Expression with its Downstream Targets in Tumour Tissues

Chapter 4 described the more likely regulation of Cten and its downstream targets (i.e., FAK and ILK) in addition to other established downstream targets, including AKT, Snail and Src by the CD24 molecule. These findings were confirmed by manipulating CD24 expression in different cancer cell line models. Accordingly, the relationship between CD24 expression and these molecules in tissues taken from colorectal and lung tumour cases was examined. Before discussing the results, it is worth mentioning that there was high background staining. The choice of controls was limited by availability and this constrains experimental interpretation.

i. Colorectal Cancer

Since the TMA was constructed from cores from the central, luminal and edge regions of colorectal tumours, the frequency of CD24 expression in these areas was evaluated. By scaling the maximum H-score at 300 and the minimum at 0, CD24 immunoreactivity staining in the above-mentioned tumour areas was observed as 300, the highest, while the lowest was 30. CD24 expression in tumour compartments was almost identical; however, a high percentage of CD24 expression was detected in central regions of tumours compared to other areas, which may be an indicator of tissue hypoxia. In matched non-tumour (normal) colonic tissue, the highest H-score was 100 and the lowest, 0.

A significant difference in CD24 expression between tumour and non-tumour (normal) ($p=0.017$) was observed, indicating that CD24 is overexpressed in

tumours. The correlation between CD24 and its downstream targets validates the *in vitro* data presented in the previous chapters.

Cten expression was associated with CD24 expression in most cases. Overall, high Cten staining was associated with 75% of high CD24 expression results. No evidence of an association between Cten expression and clinicopathological features was observed, except in a few cases, with cases of tumour staging after stage 0 being excluded. Together with previous findings, this suggests that Cten really is associated with CD24 expression. Moreover, Cten regulation by CD24 seems to take place in the cytoplasm and nucleus, suggesting their importance in cancer progression and metastasis.

As has been noted, high FAK expression was associated with 69% of high CD24 expression, while high ILK expression was associated with 63%. These observations are robustly consistent with those outlined in previous chapters, providing evidence that FAK and ILK expression are closely associated with CD24 expression. This also increases the likelihood of targeting these molecules by CD24 through Cten regulation.

It was earlier shown that CD24 is involved in AKT activation, specifically at phosphorylated sites Thr-308 and Ser473. Here, the association between the expression of AKT and CD24 in tumour tissue has been examined. Immunoreactivity staining revealed that pan-AKT (total protein) was associated with 42% of CD24 expression, compared to phosphorylated AKT (p-AKT Thr-308), which was associated with 56% of high CD24 expression; this was 14% higher than that of pan-AKT. This confirms previous findings by our group and others that showed AKT regulation by CD24. Although the expression of

p-AKT Ser473 in tumour tissue was also attempted, it was found that the anti-p-AKT (Ser473) antibody did not reveal any detectable staining, even after taking all possible troubleshooting into account during the optimization process. As a result, it was omitted from the study.

Snail expression was detected in a high percentage (80%) of tumour cases; however, only 39% of cases displayed high Snail expression. High Snail expression was found to be associated with 48% of CD24 high-intensity staining results, identifying Snail as a potential downstream target of CD24 based on this and previous observations in cancer cell line models.

With regard to Src expression, almost 33% of tumour cases showed high Src expression in both cellular compartments. It was also associated with 58% of CD24 high-intensity staining cases. Similar to previously investigated markers, Snail and Src expression in tumour cases did not show evidence of a correlation with clinicopathological features of CRC patients. However, in some cases, Src expression did correlate with tumour grade and stage.

ii. NSCLC

Similar to colorectal TMA immunostaining, CD24 expression in NSCLC tissue was detected using monoclonal CD24 antibody clone SWA11, while appropriate antibodies were used for the other markers. A few (3/58; 7%) cases showed CD24 membranous expression. Since we had three NSCLC tumour categories, the average H-score for CD24 staining intensity was determined. The highest H-score (167) was observed in carcinoid cases, while the lowest (122) was detected in SCC cases. With regard to the downstream targets of CD24, their correlations to high CD24 expression in NSCLC tissue were ranked from highest to lowest

as follows: Src: 97%; FAK and ILK: 94%; p-AKT (Thr-308): 91%; pan-AKT: 88%; Cten: 81%; and Snail: 63%.

Overall, expression of CD24 and its downstream targets in lung tumour tissue was almost equivalent to that observed in colorectal tumour tissue. However, these markers seem to be expressed more in lung tumour tissue. Since the clinicopathological features of the NSCLC tissue were missing, the correlation of expression of the studied molecules to tumour progression could not be determined. However, their expression was compared with data obtained from CRC tissue, as well as the correlation between their expression and CD24 expression. For comprehensive analysis, clinicopathological features of NSCLC tissue should be considered. Moreover, the role of CD24 as a prognostic marker and its relation to patients' death or survival should be addressed in future studies.

The marked background staining of CD24 in both CRC and NSCLC tissues highlights that the specificity of the immuno-reaction may not be full-proof. This could have been checked by peptide blocking but was not performed due to time constraints. Within the limits of interpretation, the data show that CD24 is expressed in any cellular compartment, but more dominantly in the cytoplasm.

In summary, CD24 was shown to be expressed in both the cytoplasm and nucleus in cell lines and tumour tissues. However, CD24 expression in the cytoplasm was more dominant. Patient survival was not evaluated in this study due to the lack of availability of pertinent patient data. However, previously, CD24 was shown to be a prognostic marker of patients' survival in NSCLC but not in CRC tissue.

7 GENERAL DISCUSSION

7.1 Introduction

High levels of CD24 expression have been detected in various tumours (Kristiansen et al., 2004b, Kristiansen et al., 2003b, Lee et al., 2009b, Weichert et al., 2005b). Moreover, CD24 is considered a marker of poor prognosis in some tumours (Kristiansen et al., 2003c, Kristiansen et al., 2004a, Agrawal et al., 2007). In terms of its function, CD24 has been implicated in signal transduction, as well as in the regulation of several cellular activities, suggesting that it plays a crucial role in cancer progression and metastasis (Lee et al., 2010, Kristiansen et al., 2003c). Despite a growing body of literature that identifies the significance of CD24 in cancer development, the mechanisms involved remain uncertain.

In this thesis, the signalling and biological functions of CD24 in cancer development have been investigated, focusing on two themes: a) the mechanism(s) via which CD24 regulates cell function, as determined by its potential downstream targets, such as Cten, FAK, and ILK and other related molecules identified as playing roles in cancer progression; and b) the mechanism(s) by which CD24 expression can be regulated, with a focus on identifying possible upstream regulators of CD24.

To achieve the above objectives, observations have been validated in four cancer model systems (colorectal, pancreatic, liver and lung cancer). Our work initially investigated the signalling and function of CD24 in colorectal cancer cell lines *in vitro*. However, to make the research more comprehensive, similar investigations in the other cancer models (i.e. pancreatic, liver and lung cancer) were carried out due to the common factors existing between the proprieties of

these cancers and colorectal cancer (Sagiv et al., 2008, Hahn et al., 1996, Mahlamaki et al., 2002).

Throughout this research project, the expression level of a gene of interest, often CD24, was manipulated using a dual approach consisting of forced gene expression and gene knockdown. This procedure was followed to obtain reliable results and to avoid poor or inaccurate conclusions based on the off-target effects of gene knockdown or the likely non-specific influences of forced gene expression. The findings of this study have several important implications for future practice.

7.2 Investigating the Regulatory Role of CD24 in Cancer

The localisation of CD24 on the cell surface via a GPI-anchor, binding to extracellular matrix components, and its presence in lipid rafts enable CD24 to control a variety of cellular functions (Baumann et al., 2005), thereby contributing to cancer progression and metastasis. Therefore, the effect of CD24 on the behaviour of cancer cells was studied in the four cancer models (i.e., colorectal, pancreatic, liver and lung cancer).

In these cancer models, the functional studies data showed that forced expression of CD24 in CD24-negative cell lines and knockdown of CD24 using a specific siRNA in CD24-postive cell lines resulted in changes in both cell motility and invasion. These data seem promising. However, the exact mechanism by which these cell functions are regulated by CD24 is still unclear. CD24 seems to stimulate integrin activity and, as a result, induces cell functions, including cell migration and invasion (Baumann et al., 2005). Moreover, FAK

could be an essential mediator of CD24-dependent cellular function due to its crucial role in cancer progression and metastasis (Schwartz and Assoian, 2001).

Regarding the potential role of CD24 in regulating cell proliferation in these cancer models, a number of variations in the effect of CD24 on cell proliferation were observed, specifically in CRC cell lines; thus it may not be significant in CRC. The effects observed in the other cancer models, however, suggest that CD24 regulates cell proliferation in different tumours. These functional studies, however, need to be repeated following cell treatment with mitosis inhibitors. The mechanism underlying cell proliferation regulation by CD24 in cancer is uncertain. However, previous studies reported that cell proliferation may be mediated via the Ral GTPases/CD24 pathway (Smith et al., 2006). In addition, STAT3 has been shown to be regulated by Src, which is also targeted by CD24. Taken together, these data suggest that cell proliferation is regulated through the Ral GTPases-CD24-Src-STAT3 signalling pathway. In contrast, another study suggested that CD24 is associated with the MAPK pathway in the regulation of cell proliferation (Wang et al., 2010).

Observations presented in this thesis also revealed that CD24 enhances colony formation in soft agar in different cell lines, and it may therefore confer features of stemness. However, the mechanism through which CD24 acts to stimulate the anchorage-independent growth of tumour cells "stemness" is still unknown. It probably does so by targeting Notch1, which is stabilised by p38MAPK (Lim et al., 2014) and/or via the CD24-STAT3-Src signalling pathway (Bretz et al., 2012b). The soft agar colony formation assay is an indirect measure of stemness and could be further confirmed by immunocytochemistry with putative stem cell markers.

In summary, our observations provide evidence of the significant role CD24 plays in the regulation tumour cell functions, including motility, invasion, and proliferation, and in the enhancement of certain features of the stemness phenotype model, suggesting that CD24 appears to have all the features required to promote metastasis. These findings illustrate, for the first time, that CD24 consistently regulates cell functions in different cancer model cell lines, possibly via (a) unique mechanism(s). This could contribute to the development of new therapeutic agents that could be translated into clinical practice.

7.3 Investigating the Downstream Targets of CD24 in Cancer

CD24 has been shown to have an effect on cell function in the four cancer models. Despite the huge body of literature that attempts to identify the mechanism by which these cell functions are regulated by CD24, the exact mechanism remains indistinct. The similarities found between CD24 and Cten in terms of localisation and involvement in tumour progression (Runz et al., 2008, Al-Ghamdi, 2013) have led to the hypothesis that CD24 could be an upstream effector that up-regulates Cten expression. The downstream targets of Cten, such as FAK and ILK, were also assumed to be regulated by CD24 via targeting Cten (Al-Ghamdi, 2013). Changes in the expression of the above molecules, in addition to other focal adhesion molecules including AKT and EMT markers, may be associated with tumour progression were evaluated following CD24 manipulation in appropriate cell lines using Western blot test.

a) The Mechanistic Basis of CD24-Mediated Induction of Cell Motility

i. CD24 May Regulate Cell Function Through the CD24-Cten-ILK/FAK Pathway

Manipulation of CD24 expression either by CD24 forced expression or CD24 knockdown in appropriate cell lines from the four cancer models consistently indicated that Cten, ILK and FAK expression levels are up-regulated by CD24. Changes in the expression levels of FAK and ILK have also been shown to be mirrored by modulations made in the expression level of Cten (Al-Ghamdi, 2013), suggesting a mutual CD24–Cten interaction. The likelihood of interaction between CD24 and Cten was assessed through gene co-transfection by over-expressing one of these genes and knocking down the other. CD24 and Cten were found to be associated, possibly working together to regulate cell function. This notion was confirmed by Co-IP assay, which showed that CD24 is immunoprecipitated with Cten in a protein complex. However, it is unclear whether FAK and ILK act independently or through a CD24-Cten-ILK/FAK pathway.

The potential role of CD24 in the regulation of FAK and ILK expressions via signalling through Cten was assessed. Changes in FAK and ILK expression levels were evaluated in cell lysates of SW620Cten^{KO}, a cell line that has undergone Cten knockout in our lab using CRISPR-Cas9. Neither FAK nor ILK expression level were detected, although CD24 was still expressed in this cell line. This confirms that Cten is a direct positive regulator of FAK and ILK. Similarly, cell motility and invasion were significantly affected in the absence of Cten in this cell line. Together, these data provide evidence that CD24 is a

positive effector of Cten, FAK and ILK expressions, and that it may modulate cell function through a CD24-Cten-ILK/FAK pathway.

ii. CD24 Promotes the Stabilisation of Cten Protein

Since Cten has been shown to be positively regulated by CD24, how this regulation is attained was assessed. It was assumed that this might occur as a result of mutual protein stabilization rather than mutual gene induction, based on the interaction between these molecules observed following gene-co-transfection. No effect at the mRNA level of Cten was found following manipulation of CD24 expression in suitable cancer model cell lines, suggesting that Cten expression is regulated by CD24 at the protein level. The CHX assay was used to evaluate changes in Cten protein level following appropriate transfection in suitable cell lines, followed by treatment with CHX reagent. Data showed that co-transfection with $CD24^+/Cten^+$ resulted in the stabilization of the Cten protein, while in the absence of CD24 expression in the cells (by CD24 knock down), a reduction in the expression level of Cten was observed. This finding indicates that CD24 may stimulate cell function through the stabilisation of the Cten protein. However, further investigations are required to better understand the mechanism(s) behind the up-regulation of Cten by CD24.

b) The Mechanistic Basis of CD24-Mediated Induction of Stemness

i. CD24 Induces the Epithelial-Mesenchymal Transition

Alterations in the expression levels of EMT markers, including N-cadherin, Snail and E-cadherin, were evaluated following manipulation of CD24 expression in cells of the four cancer models. The data showed that N-cadherin and Snail are positively associated with CD24 expression. However, E-cadherin

was found to be inversely associated with CD24 expression. These observations are significant, since the existence of an association between CD24 and EMT markers is important in promoting cancer cells, leading to acquisition of cancer stemness and metastasis. How EMT is regulated by CD24 is still uncertain. Stabilisation of Cten protein by CD24 suggests that CD24 could play a role in EMT processes, possibly by targeting Src and FAK signalling pathways, which have been reported to stimulate EMT (Cicchini et al., 2008).

The associations between CD24 and the expression levels of other important molecules, such as β-catenin and Src, along with a number of adaptor proteins, including Vinculin and Paxillin, were evaluated. CD24 was found to up-regulate Src expression and inhibit β-catenin expression, suggesting that Src could be another downstream target of CD24. Such a relationship between CD24 and Src may reveal the mechanism behind stemness and tumour metastasis, since Src has been reported to be involved in these processes (Desgrosellier et al., 2009). This might occur through the integrin β3/FAK signalling pathway (Desgrosellier et al., 2014). No relationship between CD24 and the expression levels of Paxillin and Vinculin was detected.

ii. CD24 Activates the AKT Pathway

Preliminary data from our group suggested that AKT is activated by CD24 in CRC cell lines (AHMED, 2011). It was also observed that CD24 phosphorylates AKT at Ser473 in the hydrophobic domain rather than at Thr308 in the activation loop. Since such a relationship between CD24 and AKT is significant in terms of regulating cellular functions in tumour cells, the above findings were validated.

Manipulation of CD24 expression in the cell lines DLD-1 and GP2D affected the expression level of p-AKT at Ser473, while no effect was seen on the expression level of p-AKT at Thr308. Consistent with preliminary data, these findings provide evidence that CD24 regulates AKT activation at Ser473. Thus, the phosphorylation site at Ser473 of AKT, rather than the Thr308 phosphorylation site, may be a model marker for AKT activity. Additionally, CD24 has also been found to regulate the activity of AKT downstream targets such as p-eNOS, p-FAK, and p-CREB. Presumably, CD24 works precisely on Ser473 and may require priming by the PI3K pathway to phosphorylate Thr308. Together, these results confirm the preliminary findings using the phosphokinome array and suggest that CD24 may induce stemness by activating the AKT pathway.

iii. Combining PI3K Inhibitor/CD24 SiRNA Enhances the Drug Activity

The effective targeting of the CD24/PI3K combination was tested, since CD24 targets AKT at Ser473 and PI3K targets AKT at Thr308. The effect of these treatments in combination or individually was evaluated using the cell lines GP2D and DLD-1. The data showed that treatments with LY294002 either at a lower dose or at a higher dose affected AKT activation at the AKT Ser473 and AKT Thr308 residues. In contrast, treatment with CD24 siRNA affected AKT activation only at the AKT Ser473 residue. In combination, CD24 siRNA and the PI3 kinase inhibitor LY294002 at both higher and lower doses resulted in an effect on AKT activation at both residues Ser473 and Thr308. However, a greater effect was observed when CD24 siRNA was combined with the AKT inhibitor at a lower dose.

The effect of similar treatments on cell motility, invasion, and proliferation was evaluated. The data showed that the effect of treatment with CD24 siRNA combined with LY294002 at a lower dose on the above cell functions was the same as that detected after treatment with LY294002 alone at the higher dose. Together, these findings suggest that combining PI3K inhibitor with CD24 siRNA may enhance the activity of the drug.

In summary, the data presented in this section suggest that CD24 may directly regulate Cten, which also regulates FAK and ILK expression, indicating that CD24 could be involved in a unique mechanistic pathway through which cell functions are regulated in the tested cancer models. Several potential downstream targets of CD24, including Cten, FAK, ILK, AKT, Src, N-cadherin, and Snail, have been identified. CD24 seems to regulate cell function through the CD24-Cten-ILK/FAK pathway. These targets may be directly regulated by CD24 or indirectly regulated through other molecules. Further studies must be conducted to reveal the exact mechanism cascade involved in CD24 signalling in cancer. Overall, CD24 seems to have features necessary to promote metastasis; however, *in vivo* studies using animal models are required to validate these data.

7.4 Investigating the Upstream Regulators of CD24 in Cancer

In light of the data that suggested that Cten is up-regulated through EGFR/KRAS signalling in colon and pancreatic cancers (Al-Ghamdi et al., 2011), along with the observations generated in this research project showing that CD24 up-regulates Cten, it was hypothesised that EGFR/KRAS signalling

positively regulates CD24 signalling and that CD24 in turn positively regulates Cten.

A Mutual Inhibitory Relationship between KRAS and CD24

Establishing an association between KRAS signalling and CD24 expression could reveal a novel regulatory mechanism by which Cten is controlled by KRAS signalling through CD24. Data obtained by our group showed that KRAS inhibits CD24 expression. Moreover, CD24 was found to inhibit KRAS expression. Together, these data suggest a mutual inhibitory relationship between KRAS and CD24. In addition, these findings suggest that KRAS seems to not target Cten through CD24 but probably through other signalling pathways, such as the STAT-JAK complex cascade, as reported in a previous study (Andl et al., 2004), or through STAT3, which has been found to inhibit Cten expression (Kwon et al., 2011) but up-regulates CD24 (AHMED, 2011).

EGFR is an Upstream Regulator of CD24

Since KRAS is an important component of the EGFR pathway, the possible relationship between CD24 and EGFR, as a potential upstream regulator, was studied through the inhibition of EGFR activity with a specific EGFR tyrosine kinase inhibitor (PD135053) and following stimulation of EGFR activity with recombinant-EGF in appropriate cell lines that were confirmed as wild-type for KRAS. The expression levels of KRAS, CD24 and Cten were observed to be positively up-regulated by EGFR. The change in cell motility shown by the Transwell migration assay was probably modulated by the EGFR-CD24-Cten pathway. Together, these findings suggest that EGFR is an upstream regulator of KRAS, CD24 and its downstream targets (e.g. Cten). However, CD24 is

apparently up-regulated by EGFR either directly or via other signalling pathways (e.g. PI3K) but not through KRAS.

To validate the above data, EGFR activity was stimulated with recombinant EGF PHG0333L at a concentration of 20 ng/ml in appropriate cell lines, which resulted in a change in the expression levels of KRAS, CD24 and Cten. Similarly, such a treatment resulted in a significant change in cell motility. Together, these observations suggest that EGFR plays a vital role in the regulation of cell function, potentially by targeting CD24. To our knowledge, this is the first time that EGFR has been suggested as an upstream regulator of CD24. Overall, a new potential upstream regulator of CD24 (i.e. EGFR) has been successfully identified, yielding an EGFR-CD24-Cten signalling pathway.

7.5 Investigation of the Subcellular Localisation of CD24

The Subcellular Localisation of CD24 in Tumour Cell Lines

The subcellular localisation of CD24 in cancer cells was studied as limited studies have shown its expression in these compartments, specifically in the nucleus. The data from Western blotting performed after protein fractionation of appropriate cell lines showed that CD24 localised to both the cytoplasm and the nucleus. Control antibodies confirmed that the protein fractionation procedure had been carried out successfully and that the results obtained were accurate. These observations were robustly consistent with previous published data in CRC (Weichert et al., 2005a) and in NSCLC (Kristiansen et al., 2003a). Nuclear CD24 expression may suggest that CD24 physically crosses the threshold into the nucleus, although this remains unclear. These observations imply that CD24 up-regulates Cten, which is principally localised within the nucleus, suggesting

involvement of CD24 in Cten regulation in subcellular compartments, thereby contributing to tumour progression and metastasis (Albasri et al., 2011).

The Subcellular Localisation and Expression of CD24 in Tumour Tissues

The expression of CD24 was immunohistochemically evaluated in tissues from 84 CRC and 58 NSCLC cases using a CD24 monoclonal antibody (clone SWA11). In both CRC and NSCLC tissues, the cytoplasmic and nuclear expression of CD24 was detected. In all cases, the ratio of cytoplasmic CD24 expression to nuclear CD24 expression was higher in CRC than in NSCLC. The localisation of CD24 in the nucleus was previously shown in CRC. However, this is the first time nuclear CD24 localisation has been established in NSCLC. These findings suggest that nuclear CD24 expression is a phenomenon, since this has been confirmed in two different cancer models using two different techniques. CD24 expression in subcellular compartments in CRC tissue did not correlate with clinicopathological features, except in a few cases in which CD24 nuclear overexpression significantly correlated with the presence of lymph-node metastasis and more advanced pathological stages. In NSCLC, the clinicopathological features were not available.

Association of CD24 Expression with its Downstream Targets in Tumour Tissues

To confirm the association between CD24 expression and its downstream targets (i.e. Cten, FAK, ILK, AKT, Snail, and Src) established following modulation of CD24 expression in different cancer model cell lines, the expression of these molecules in tissues taken from colorectal and lung tumour cases was immunohistochemically studied.

a. In CRC Tissue

CD24 immunoreactivity staining in the central, luminal and edge regions was detected as 300, the highest, while the lowest was 30. CD24 expression in tumour regions was almost equal; however, a high level of CD24 expression was identified in central regions of tumours, possibly indicating hypoxic tissue.

High CD24 expression was strongly associated with high expression of its downstream targets, ranging from 48% (Snail, the lowest) to 76% (Cten the highest). These findings are consistent with those obtained in the cancer model cell lines following manipulation of CD24 expression, and confirm the up-regulation of Cten, FAK, ILK, AKT, Src and Snail by CD24. Cten regulation by CD24 may occur in both the cytoplasm and nucleus, leading to cancer progression and metastasis.

b. In NSCLC Tissue

Since different types of lung tumour were included in this study, the average H-score for CD24 staining intensity was determined. The highest H-score of CD24 immunostaining was found in carcinoid cases (167), while the lowest (122) was noted in SCC cases. Compared to observations in CRC tissue, the association between the high expression of CD24 and its downstream targets was higher in NSCLC tissue, ranging from the lowest (Snail 63%) to the highest (FAK, 97%).

In summary, although IHC techniques have some limitations, including reproducible artefacts due to binding to other epitopes/molecules, CD24 localisation in subcellular compartments was confirmed to be a phenomenon as CD24 expression has been seen in both the cytoplasm and nucleus in cell lines

as well as in tumour tissues. Consistent with observations in the cancer model cell lines, a high association between the expression of CD24 and its downstream targets was detected in both CRC and NSCLC tissues.

The marked background staining of CD24 in both CRC and NSCLC tissues highlights that the specificity of the immuno-reaction may not be full-proof. This could have been checked by peptide blocking but was not performed due to time constraints.

7.6 Conclusions

In this study, CD24 signalling and functions have been investigated in four cancer models, which consisted of colorectal, pancreatic, liver, and lung cancers. Observations obtained following the manipulation of CD24 via either CD24 forced expression or knockdown showed that CD24 is involved in the regulation of cell functions, including motility, invasion, and proliferation as well as in the enhancement of cancer stemness features. This may occur via signalling through multiple downstream targets, including Cten, FAK, ILK, AKT, Snail, Src and N-cadherin. In addition, a close relationship between the CD24 and Cten signalling pathways was found, suggesting that they interact with and regulate the expression of one another.

EGFR was also found to up-regulate CD24 expression, resulting in the up-regulation of Cten through other signalling pathways but not through KRAS. The EGF-CD24-Cten-ILK/FAK pathway has been successfully identified, that may reveal the mechanism by which CD24 acts in tumour diseases. Furthermore, CD24 was shown to influence AKT, which may be its basis of stemness.

Finally, the subcellular localisation of CD24 has been confirmed in tumour cell lines, as well as in tumour tissues. The association between CD24 and its downstream targets in tumour cells has been validated using tumour tissues for immunohistochemical studies.

7.7 Potential Strengths of the Study

1. The experimental work performed throughout the research project was undertaken using four cancer models: colorectal, pancreatic, liver and lung cancer.
2. Several well-characterised cell lines from the above-mentioned cancer models were used for the relevant investigations, and most of the studies were performed using at least three cell lines.
3. A dual approach consisting of gene ectopic expression and knockdown was employed to investigate the signalling and functional features of CD24 and its associated molecules.
4. The stabilisation of Cten protein by CD24 and a potential interaction between their expressions has been reported for the first time.
5. In this study, multiple downstream targets of CD24 including Cten, FAK, ILK, AKT, Snail, Src and N-cadherin, were identified.
6. For the first time, this study also confirmed that CD24 regulates cell functions through Cten, which itself targets FAK and ILK.
7. We also showed that CD24 is positively regulated by EGFR; however, further investigation is required to elucidate the mechanism involved.
8. The preliminary results showing a potential association between CD24 and AKT were further validated using immunoblotting and specific antibodies.
9. CD24 has been observed to be localised to the cytoplasm, as well as in the nucleus, in tumour cell lines and tumour tissues.
10. This study is the first to confirm the association between CD24 and its associated molecules in tumour cell lines and in tumour tissues by using the anti-CD24 antibody (clone SWA11) to detect CD24 expression.

7.8 Potential Limitations of the Study

1. Because of the diversity of the used cell lines, the transfection conditions required a long time to optimise.
2. More functional studies should have been performed to study CD24's effect on cell differentiation and cell-adhesion.
3. CD24 signalling and its effect on cell behaviour in liver cancer were studied in only a single cell line.
4. In some experiments, transfection efficiency was only evaluated using Western blotting.
5. The subcellular localisation of CD24 should have been studied using the immunofluorescence technique.
6. The marked background staining of CD24 in tumour tissues highlights that the specificity of the immuno-reaction may not be full-proof. This could have been checked by peptide blocking but was not performed due to time constraints.
7. The soft agar colony formation assay is an indirect measure of stemness and could be further confirmed by immunocytochemistry with putative stem cell markers.
8. The effect of CD24 on cell functions, in addition to its potential role in cancer metastasis, was only studied *in vitro.* Similar studies should be conducted *in vivo* to confirm our observations in this study.

7.9 Recommendations and Future Work

1. An investigation into the relationship between CD24 and P-selectin, as well as other potential ligands, should be performed.
2. The effect of endocytosis inhibitors (e.g., chlorpromazine, nystatin, and indomethacin) on CD24 expression as well as on cell behaviour should be studied subsequent to the disruption of lipid raft in various cancer models.
3. The role of CD24 in the nucleus must be studied further.
4. An investigation into the potential connection between CD24 expression and mutant biomarkers in cancer cell lines, as well as the effect of such a relationship on cell functions, should be performed.
5. The relationship between the established upstream regulators and downstream targets of CD24 should be further studied to determine the exact mechanism via which CD24 regulates cell functions.
6. It is important to validate the molecular and functional interactions between EGFR, CD24 and Cten *in vivo* using animal models, specifically using cells that have been stably transfected with the indicated siRNA.
7. The effect of CD24-siRNA, along with LY294002 AKT inhibitor, on cell functions could be translated into clinical practice for therapeutic purposes. To achieve this objective, our observations should be validated in cell lines from various cancer models.
8. The effect of CD24 expression, together with other cancer stem cell markers, such as CD133 and CD44, on cell functions should be evaluated *in vitro* and *in vivo* in the same cancer models used in this study.

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9 APPENDICES

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                  60        70        80
TNATTKAAGG ALQSTASLFV VSLSLLHLYS
```

Figure 9-1. The CD24 protein sequence, Ref. P25063 (CD24_HUMAN).

```
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//
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Figure 9-2. Homo sapiens CD24 molecule (CD24), mRNA. NCBI Reference Sequence: NM_013230.3**.**

Locus NM_013230 2513 bp mRNA linear PRI 16-SEP-2016.

DEFINITION Homo sapiens CD24 molecule (CD24), transcript variant 1, mRNA.

SOURCE Homo sapiens (human) chromosome="6" /map="6q21"

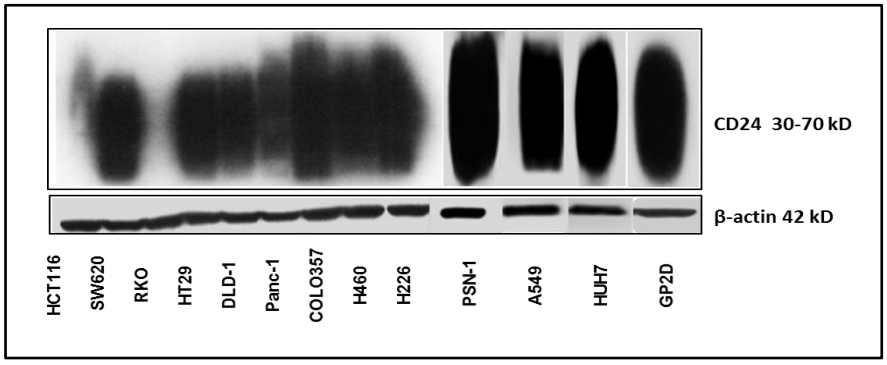

Figure 9-3. Western blot screening of CD24 expression levels in cancer model cell lines. CD24 expression levels in cancer model cell lines, CRC (HCT116, SW620, RKO, DLD-1 and HT29), pancreatic (Panc-1, COLO357 and PSN-1), Non-small cell lung cancer (H226, H460 and A549) and liver (HUH-7) were evaluated using a NuPAGE gradient (7-12%) gels. Anti-CD24 antibody clone SWA11 was used to detect CD24 expression levels, while β-actin was used as a loading control protein. Anti-mouse antibody was used as a secondary antibody.

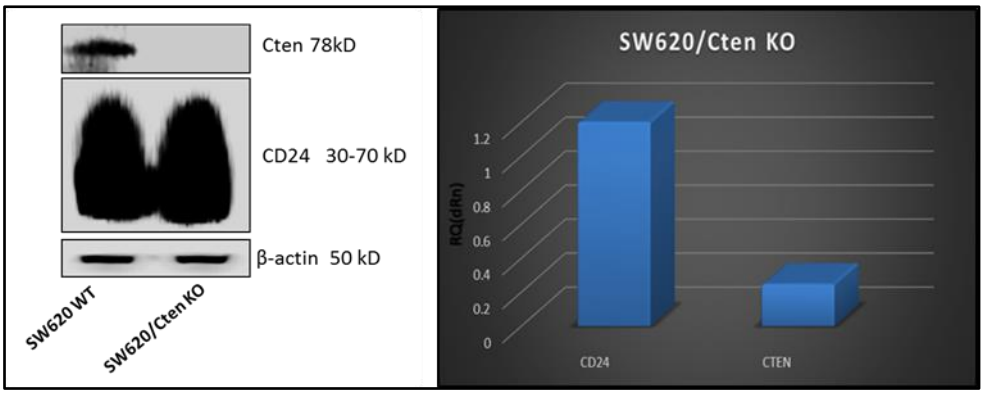

Figure 9-4. The expression levels of CD24 and Cten in SW620^{CtenKO}. This cell line shows high CD24 and absent Cten following Cten knockout using CRISPR-Cas9 as evaluated by Western blotting and qPCR.

Table 9-1. Biomarkers tested for mutation profile of cell lines.

Marker	Gene Type	Exons
BRAF	Oncogene	11 & 15
KRAS	Oncogene	3 & 4
PIK3CA	Oncogene	9 & 20
PTEN	Oncogene	3 & 8
SMAD4	Gene suppressor	9 & 11
TP53	Gene suppressor	2& 6

Table 9-2. Mutation profile of cell lines.

	KRAS	KRAS	BRAF	BRAF	PIK3CA	PIK3CA	PTEN	PTEN	TP53	TP53	SMAD4	SMAD4
	Exon 3	Exon 4	Exon 11	Exon 15	Exon 9	Exon 20	Exon 3	Exon 8	Exon 2	Exon 6	Exon9	Exon11
HCT116	WT	WT	WT	WT	WT	Mutant	WT	WT	Mutant	Mutant	WT	WT
RKO	WT	WT	WT	Mutant	WT	Mutant	WT	WT	WT	WT	WT	WT
SW620	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
DLD1	WT	WT	WT	WT	Mutant	WT	WT	WT	WT	WT	WT	WT
G2PD	WT	WT	WT	WT	WT	Mutant	WT	WT	WT	WT	WT	WT
PSN-1	WT	WT	WT	WT	WT	WT	WT	Mutant	WT	WT	Mutant	WT
Panc-1	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
A549	WT	WT	WT	WT	WT	WT	WT	Mutant	WT	WT	WT	WT

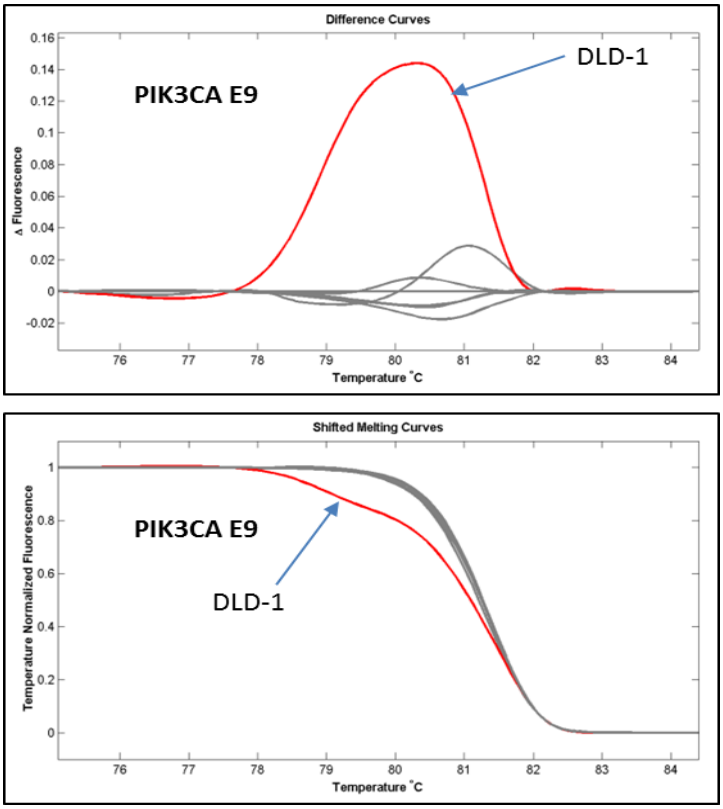

Figure 9-5. Mutated *PIK3CA* E9 in DLD-1 cell line.

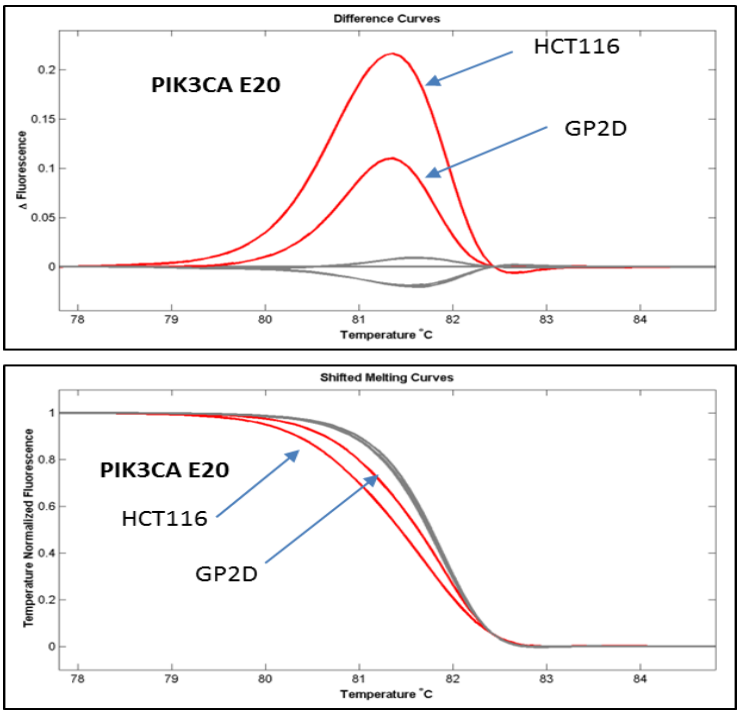

Figure 9-6. Mutated *PIK3CA* E20 in HCT116 and GP2D cell lines.

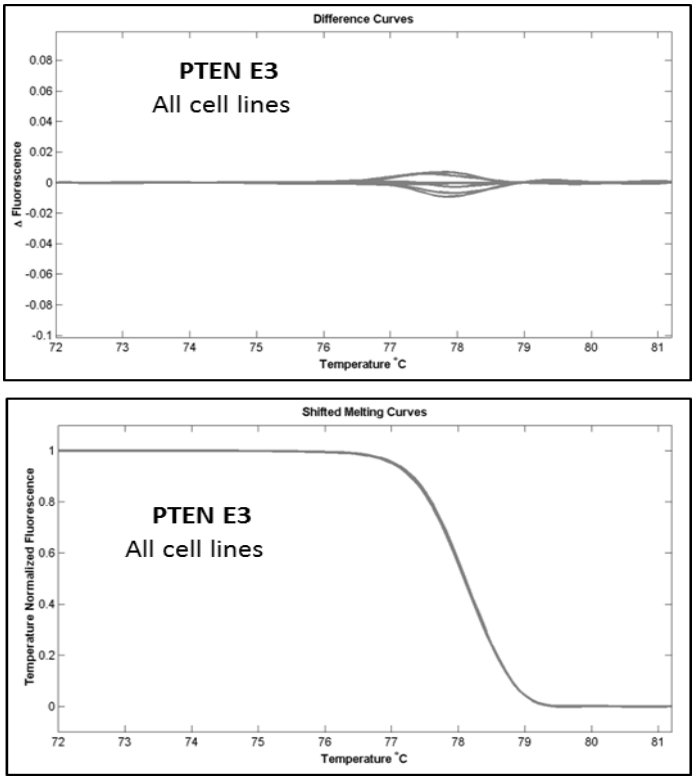

Figure 9-7. Wild-type *PTEN* E3 in cell lines.

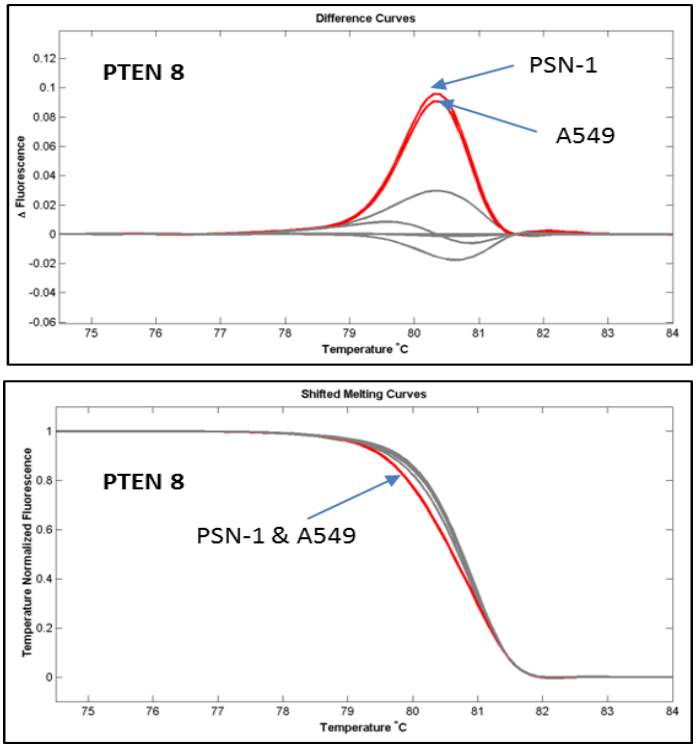

Figure 9-8. Mutated *PTEN* E8 in PSN-1 and A549 cell lines.

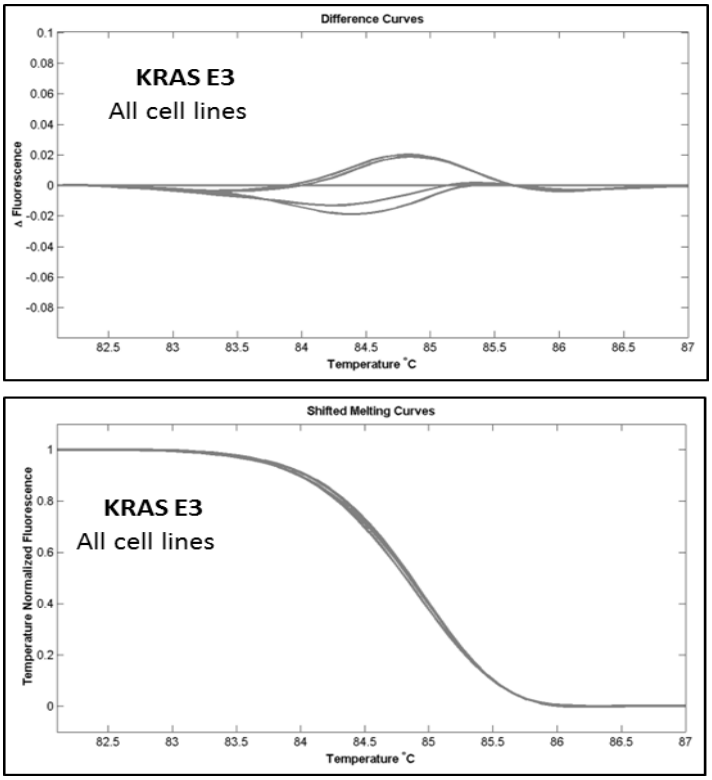

Figure 9-9. Wild-type *KRAS* E3 in cell lines.

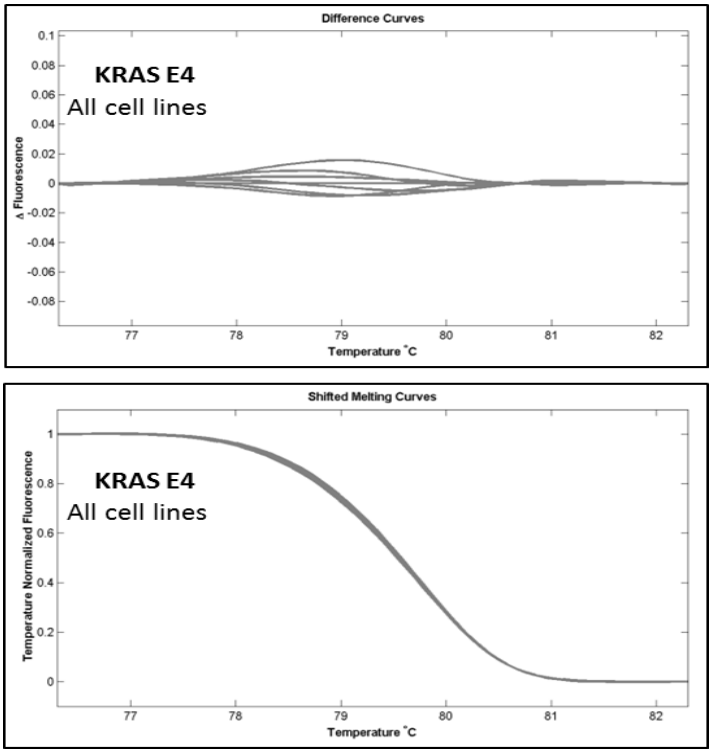

Figure 9-10. Wild-type *KRAS* E4 in cell lines.

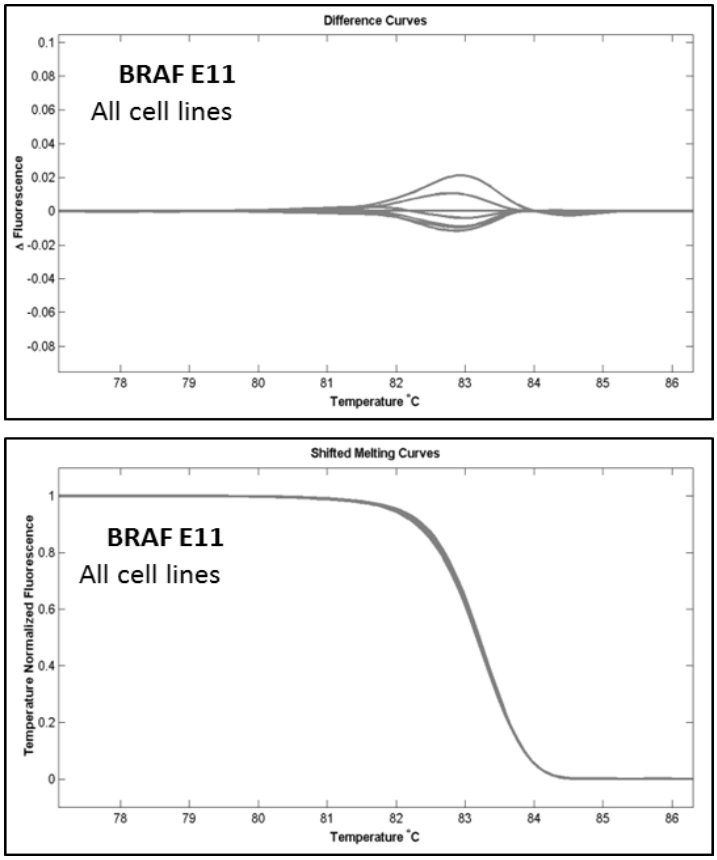

Figure 9-11. Wild-type *BRAF* E11 in cell lines.

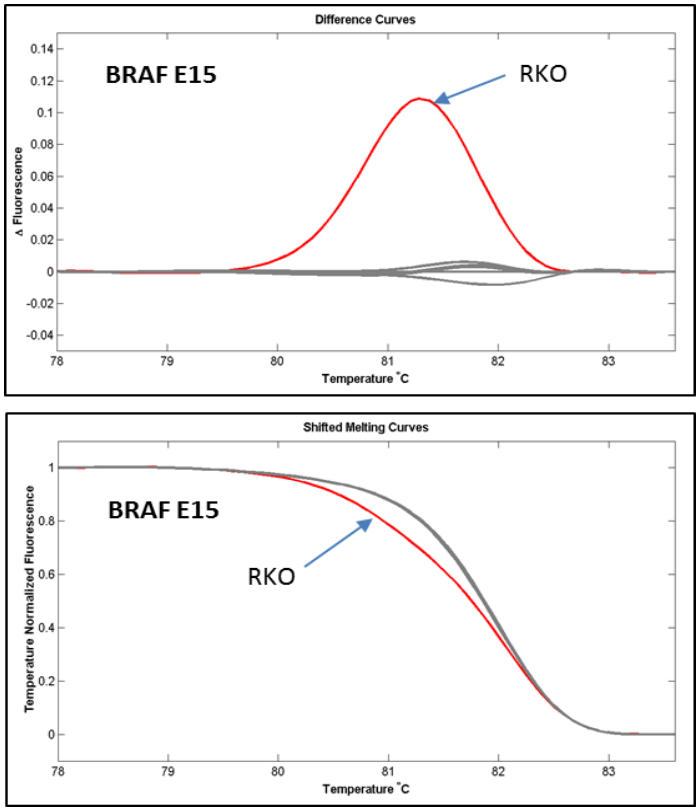

Figure 9-12. Mutated *BRAF* E15 in RKO cell line.

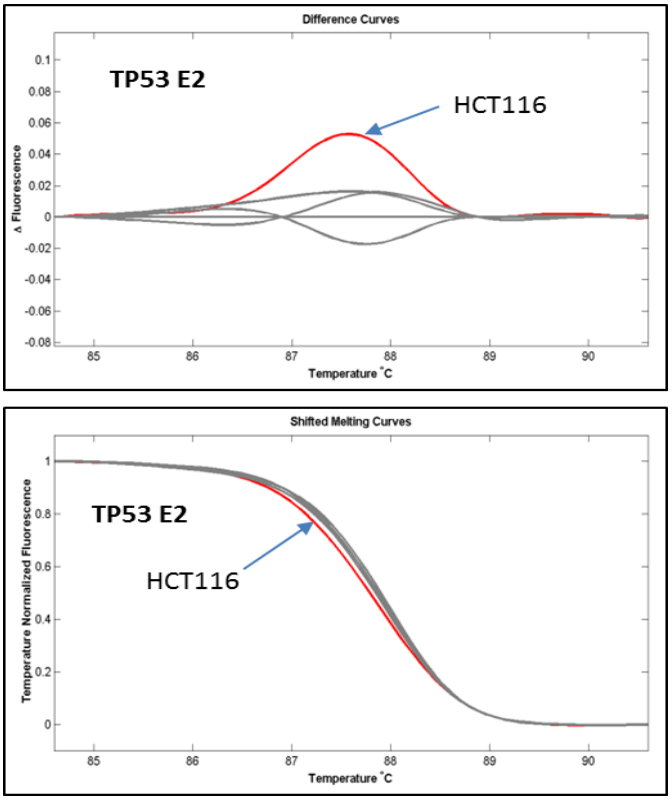

Figure 9-13. Mutated *TP53* E2 in HCT116 cell line.

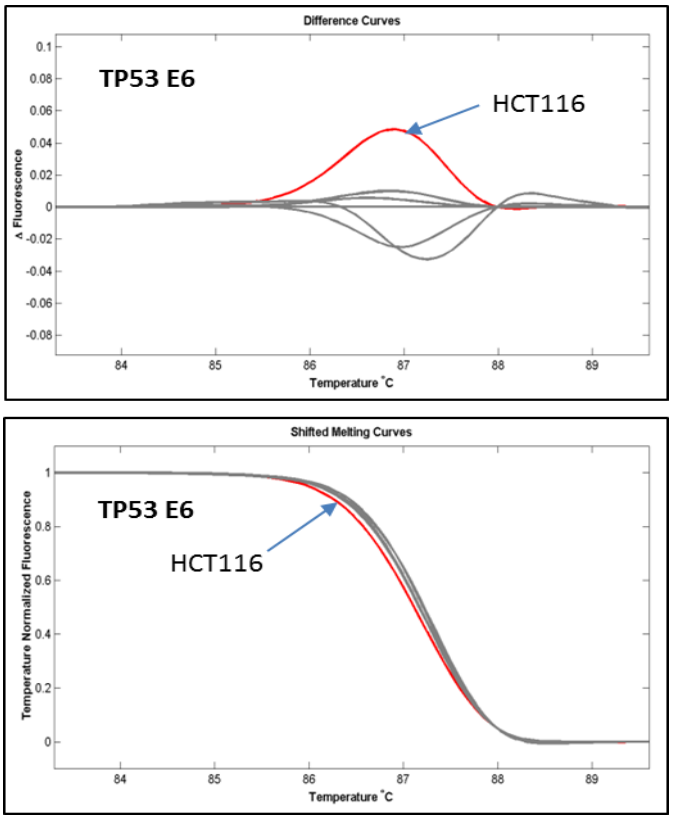

Figure 9-14. Mutated *TP53* E6 in HCT116 cell line.

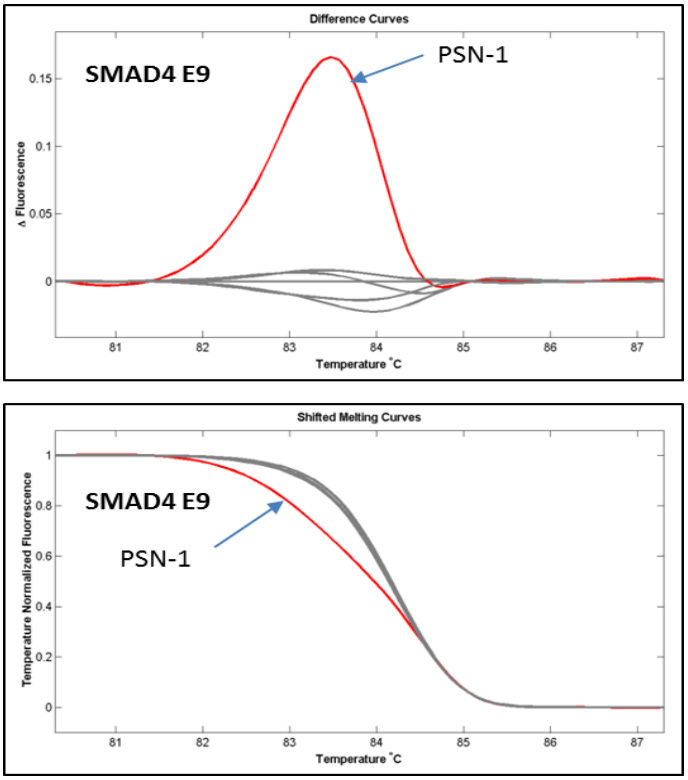

Figure 9-15. Mutated *SMAD4* E9 in PSN-1 cell line.

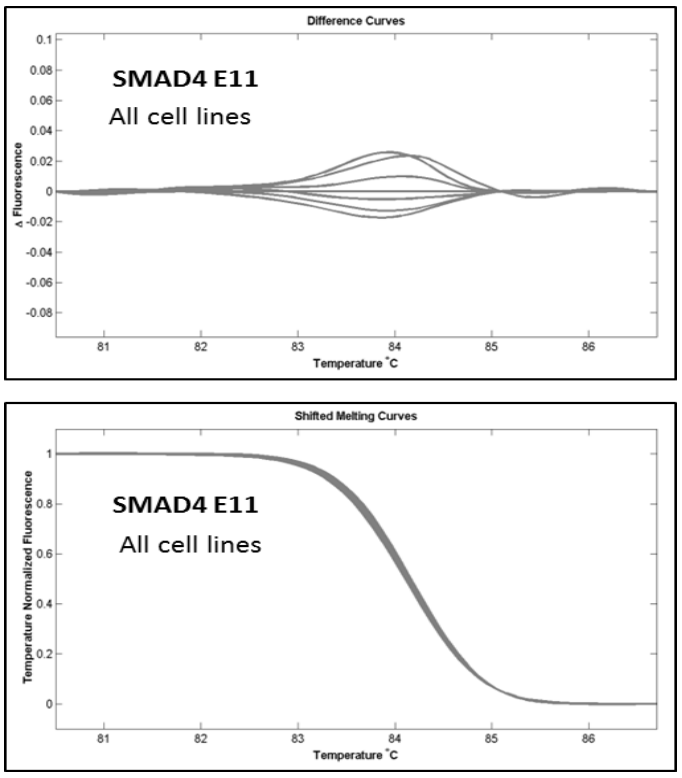

Figure 9-16. Wild-type *SMAD4* E11in cell lines.

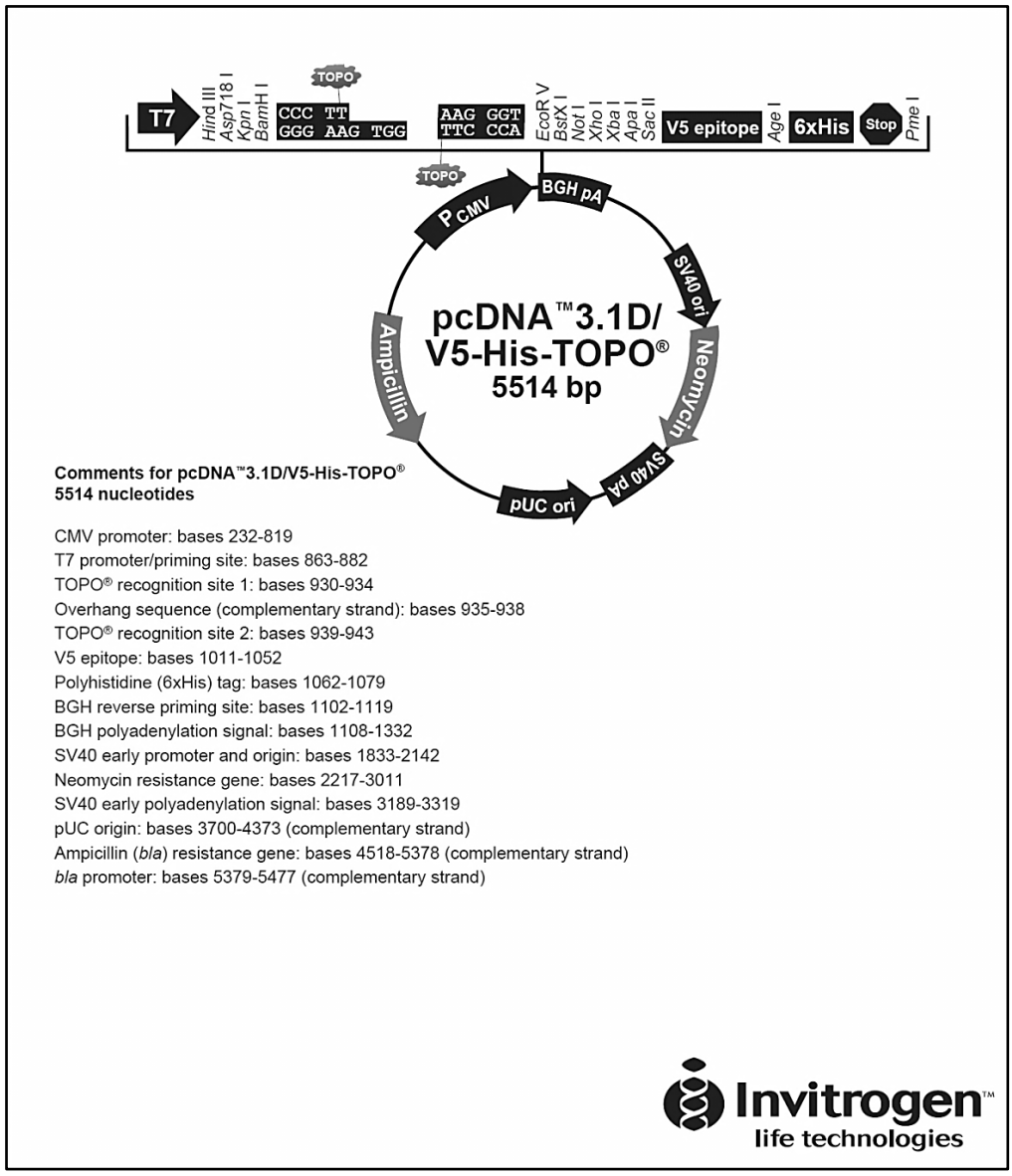

Figure 9-17. pcDNA™3.1d/v5-His-TOPO® vector map.

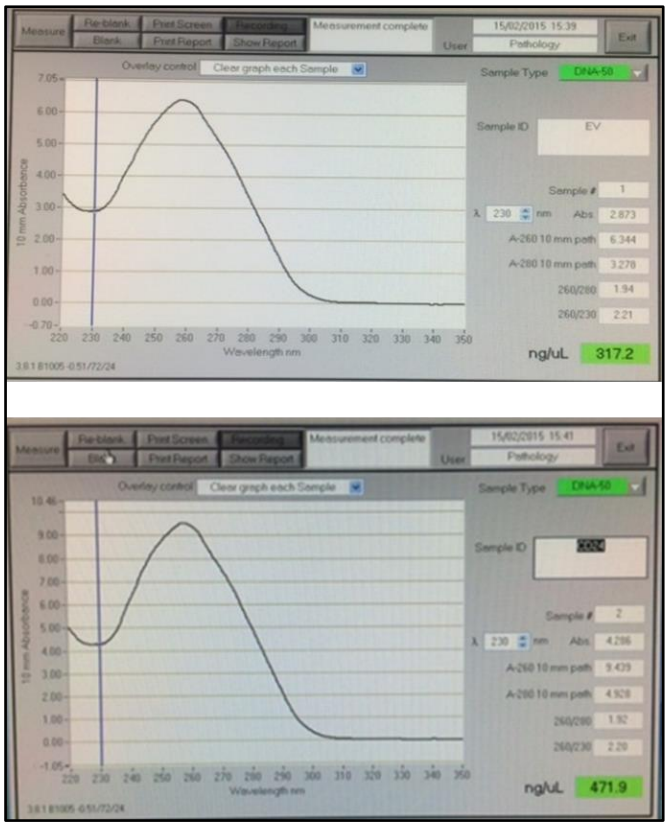

Figure 9-18. The plasmids concentrations measured using NanoDrop Spectrophotometer. Empty vector (upper), and CD24 (lower).

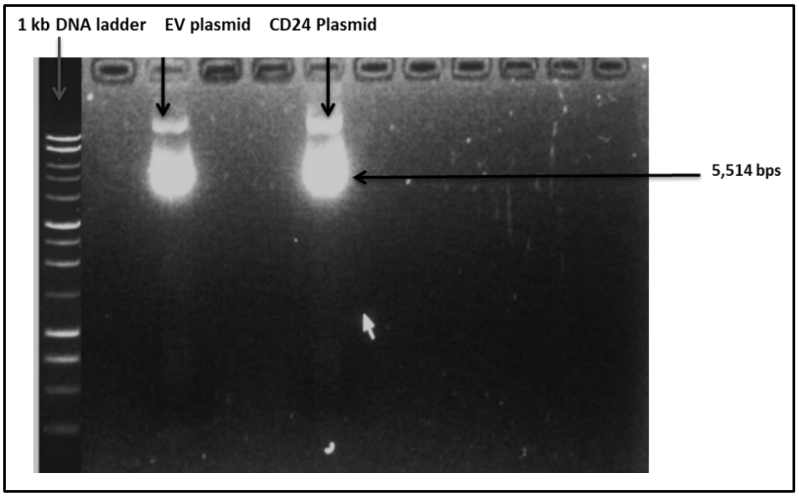

Figure 9-19. Evaluation of DNA plasmids and the efficiency of transformation. To check the efficiency of bacterial transformation, the DNA plasmids were evaluated using 2% agarose gel. After purifying DNA plasmids, the left hanged band of EV and the right band of CD24 plasmid were found have the same size of 5,515 bp.

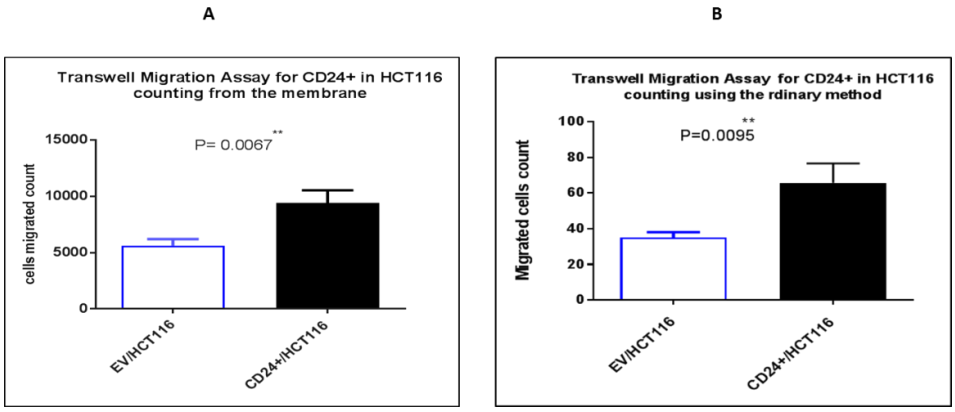

Figure 9-20. Methods used for cell counting in Transwell migration and invasion assays. **A.** In this method, cells were swabbed from the upper part of the membrane, treated with trypsin-EDTA for 10 min, re-suspended with media and then counted using the automated cell counter. **B.** The standard method, cells were counted in different fields under the microscope. Figures show the accuracy of the first (A) method compared to the standard one (B).

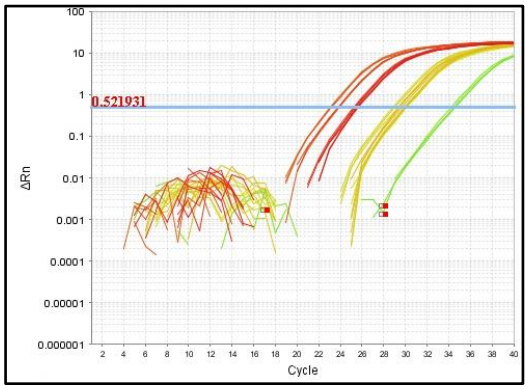

Figure 9-21. Cten amplification plot.

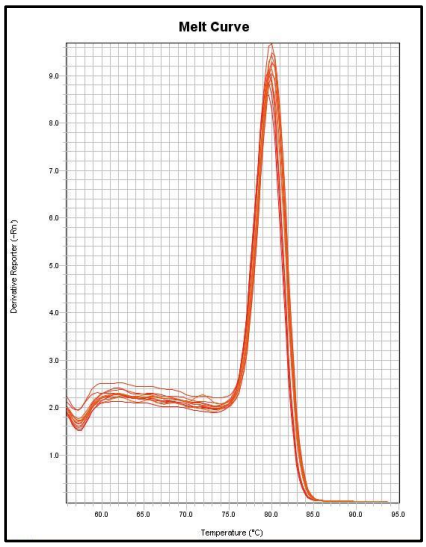

Figure 9-22. Cten melt curve.

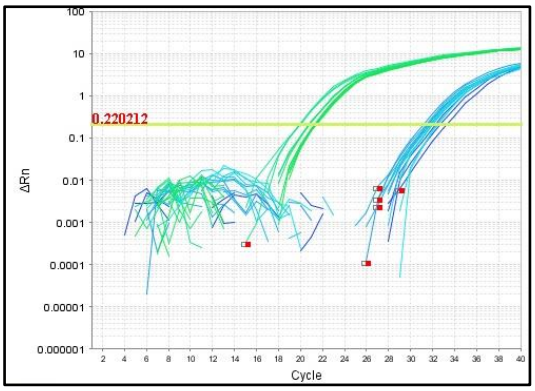

Figure 9-23. HPRT amplification plot.

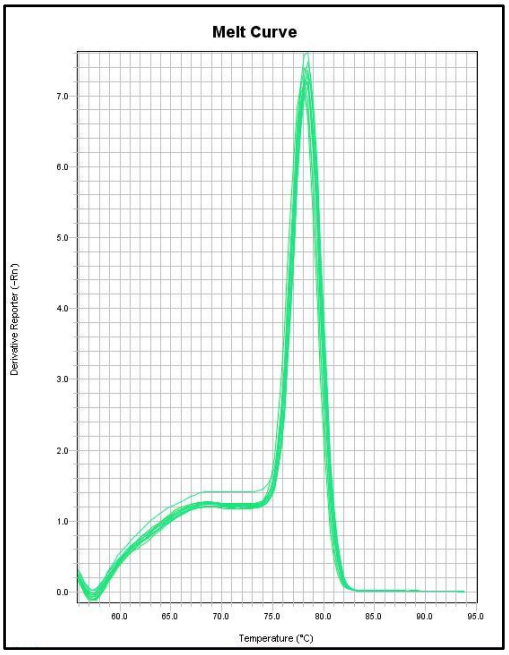

Figure 9-24. HPRT melt curve.

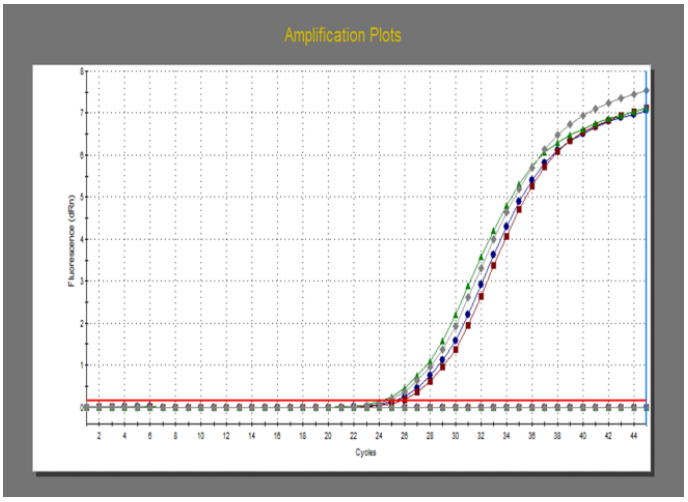

Figure 9-25. HPRT amplification plot.

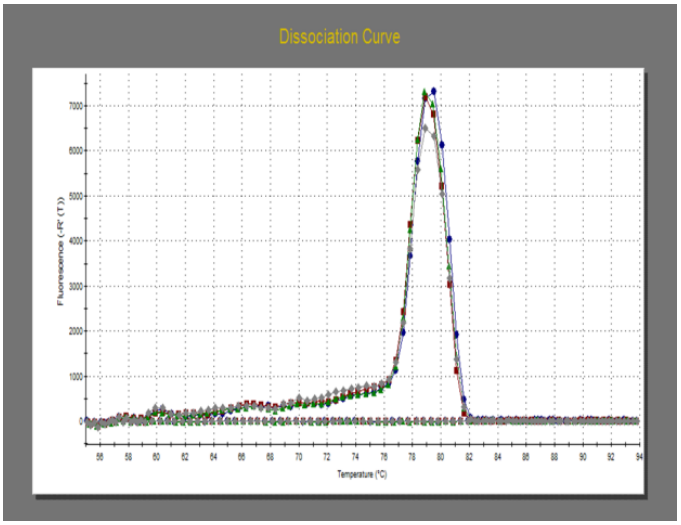

Figure 9-26. HPRT dissociation curve.

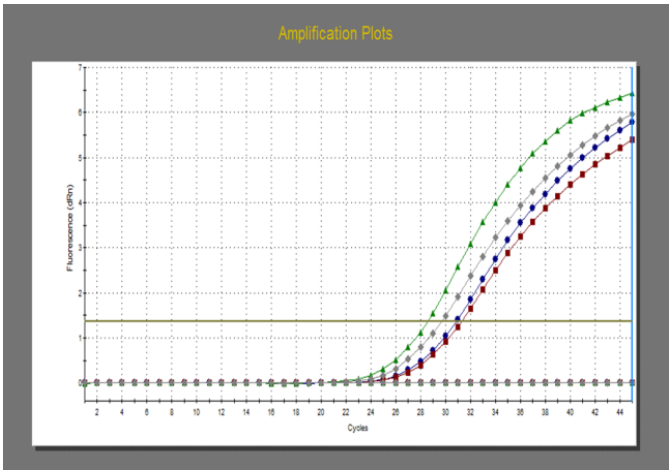

Figure 9-27. Cten amplification plot.

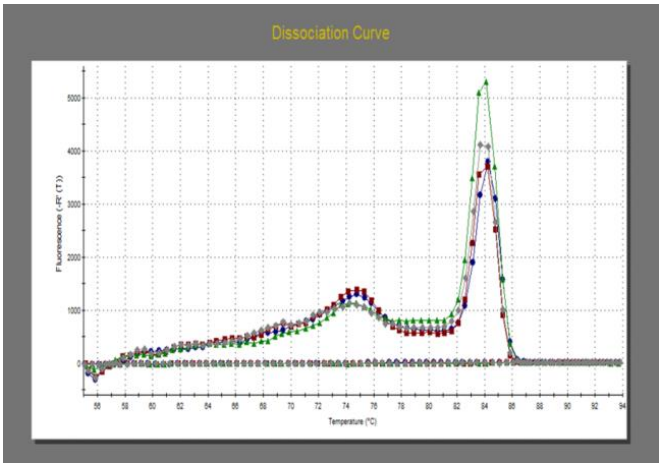

Figure 9-28. Cten dissociation curve

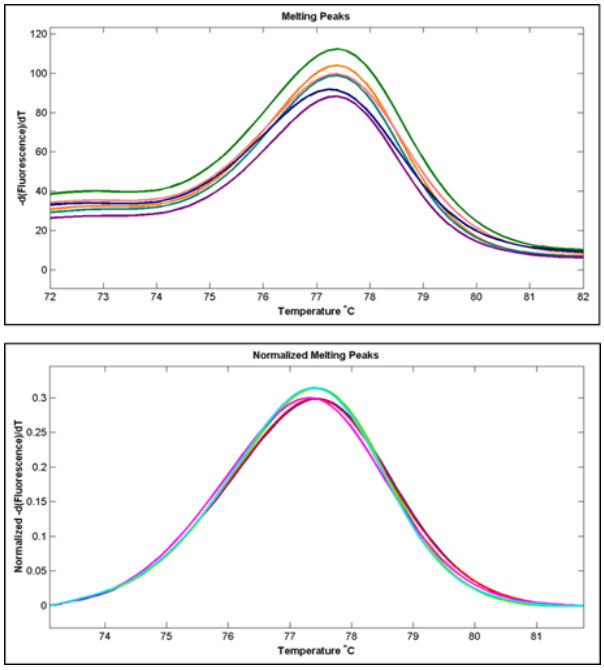

Figure 9-29. HRM for the gradient temperature of Cten primers. The gradient PCR ranged from 50 to 60°C for each primer. The data were analysed using HRM. The actual optimal annealing temperature for the reaction was 55°C.

Table 9-3. Antibodies used for IHC staining.

Antibody	Supplier	Optimized concentration
CD24 SWA11	Gifted	1:200
Cten	Sigma	1:100
FAK	Cell signalling	1:20
ILK	Cell signalling	1:10
Src	Cell signalling	1:50
Snail	Cell signalling	1:30
pan-AKT	Cell signalling	1:100
p-AKT Thr 308	Cell signalling	1:40